Louisiana State University
LSU Digital Commons

LSU Doctoral Dissertations | Graduate School

6-25-2016

Discovering Potential Protein, Carbohydrate, and Lipid Based Food Ingredients in a Co-Culture of Microalgae

Chelsea Tyus
tyuscm@yahoo.com

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_dissertations

 Part of the Agricultural Science Commons, Food Biotechnology Commons, and the Food Chemistry Commons

Recommended Citation
Tyus, Chelsea, "Discovering Potential Protein, Carbohydrate, and Lipid Based Food Ingredients in a Co-Culture of Microalgae" (2016). *LSU Doctoral Dissertations*. 5018.
https://digitalcommons.lsu.edu/gradschool_dissertations/5018

This Dissertation is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Doctoral Dissertations by an authorized graduate school editor of LSU Digital Commons. For more information, please contactgradetd@lsu.edu.

DISCOVERING POTENTIAL PROTEIN, CARBOHYDRATE, AND LIPID BASED FOOD INGREDIENTS IN A CO-CULTURE OF MICROALGAE

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The School of Nutrition and Food Sciences

by
Chelsea Tyus
B.S., Alabama A&M University, 2009
M.S., University of Missouri, 2014
August 2019

Copyright © 2019

Chelsea Tyus

All Rights Reserved.

This dissertation is dedicated to my loved ones and fellow women scientists, especially women scientists of color who must fight for a seat at the table and work twice as hard to stay there.

To my family thank you for supporting me and giving me wings so that I could believe in myself and achieve this goal. Sincere thanks to my parents, Thomas & Sherry Ousley, who covered every expense they could with no complaints, provided me sound advice, gave me love and a great foundation. Dad thanks for starting the Infinite Scholar Program and showing me what responsibility, dedication and sacrifice look like. Mom thanks for believing in me, traveling to see me in Louisiana whenever you could, and for calling every night so I could never feel lonely. To my maternal grandmother, Martha Wilcox, a million thanks for calling me every morning to wake me up and make sure I was going to school, for listening to me complain, for caring about my friends, for making me laugh endlessly, for loving me unconditionally. I thank God that you're my grandmother and my friend. To my late paternal grandmother, Verneda Tyus, I love you and have fond memories of the times we shared. To my best friends thank you for being supportive, comedic relief, sounding boards, editors, and traveling companions. To my angel, Melisa Mershon, thank you for spiritual guidance, laughs and endless encouragement. You impacted the woman I am greatly and will be missed.

I hope that me getting this degree inspires my family, friends and community to chase after their dreams. I never imagined my education would take this route. Thank you, Alabama A&M University, and University of Missouri for turning me into a great student and scientist. I am thankful for the journey, growth, opportunity, and perseverance.

The most beautiful experience we can have is the mysterious. It is the fundamental emotion that stands at the cradle of true art and true science.

-- Albert Einstein
The World as I See It

Acknowledgments

I would like to thank Dr. Joan King for accepting me as a Ph.D. student in her lab group within the LSU School of Nutrition and Food Sciences, she is a patient, understanding and knowledgeable advisor. Dr. King has propelled me to new heights in research and leadership. I would like to thank my graduate committee members Dr. Zhimin Xu, Dr. Maria Teresa Gutierrez-Wing and Dr. Jong Ham for their time and expertise. Special thanks to Nick Magazine, JeeWon Koh, Millicent Yeboah-Awudzi, Gabriella Paz, and Kwan Rhea for their laboratory expertise in food science, their comradery and shoulders to cry on. Thank you to Dr. BeiBei Guo, Connie David, Dong Liu, Dr. Gauthier, Dr. Jeonghoon Lee, Dr. Jack Losso and his lab, LSU Soil Science Lab, and LSU Aquatic Germplasm and Genetic Resources Center for lab analysis and guidance. I would like to thank my fellow lab mates for encouragement and lab assistance. I would like to thank Petrie Baker, Mitch Boudreaux, Sue Hagius, Celika Murphy, and Jennifer Marceaux for always being helpful, responding to emails, scheduling rooms, and answering every question I threw at them over the years. I would like to thank Catherine McKenzie for her help with formatting this dissertation. I have immense gratitude for IFT, IFTSA, AOCS, Phi Tau Sigma Honor Society, ABO, CABLE program (Melinda Lloyd, Denny Hall, Dr. Giovanna Aita), Louisiana Fish Fry Products, Inc. (Tana Pittman, Erika Hall), Sugar Belle (Kasie Coleman) for networking, work experience, leadership and guidance.

Finally, a thank you to Louisiana State University and the LSU Board of Regents for funding/allowing me to achieve this goal in my academic career. I am proud to say I am a graduate of the prestigious Louisiana State University and Geaux Tigers! While at LSU I was awarded many great, opportunities, friendships and experiences that allowed me to grow as a person and as a food scientist.

Table of Contents

List of Tables

List of Figures

Abbreviations

CCA	Louisiana Native Co-Culture of Microalgae (*Chlorella Vulgaris L.* and Cyanobacteria *Leptolyngbya* sp.)
Chl	*Chlorella Vulgaris L*
Cya	Cyanobacteria *Leptolyngbya sp*
ASI	average scalar irradiance
TRT	treatment
DWB	dry weight basis
PAR	photosynthetically active radiation
LPM	liters per minute
LPS	liters per second
AVG	average
STD. DEV	standard deviation
TPC	total protein content
AA	amino acid
TCA	trichloroacetic acid
TS	total starch
NRS	non-resistant starch
RS	resistant starch
PUFA	polyunsaturated fatty acid
FAME	fatty acid methyl ester

Abstract

Louisiana Native Co-Culture of Microalgae (*Chlorella Vulgaris L.* and Cyanobacteria *Leptolyngbya* sp.) (CCA) was studied. CCA is a viable coculture for further investigation as a food component. This research characterized the proteins, carbohydrates, and lipids present in CCA. Algae cultivation parameters were controlled and analyzed. Treatment (Trt) 1 was CCA grown in cultures exposed to average scalar irradiance (ASI) of 1041 ± 269.18 μmol m-2 s-1 PAR and trt 2 was CCA grown in cultures exposed to ASI of 430 ± 96.03 μmol m-2 s-1 PAR. The trt (irradiance exposure) had the desired response on CCA species ratio Trt 1 yielded an average culture ratio of 97.47 ± 1.29% Chl, and 2.84 ± 1.27% Cya. Trt 2 yielded an average culture ratio of 89.85 ± 1.17 Chl, and 10.64 ± 1.97 Cya. Total protein content was 29.46 ± 6.11 and 39.69 ± 5.15 g protein per 100 g of algae DWB for trts 1 and 2 respectively ($p = 0.001$). Total sugar content (TSC) was calculated as 25.44±6.90 g/100 for trt 1 (71% of Treatment 1 CCA's carbohydrates are starch, comprised of 23% resistant starch (RS), and 48% non-resistant starch (NRS)). TSC for trt 2 was 19.28±2.84 g/ 100g (82% of trt 2's carbohydrates are starch, comprised of 26% RS, and 56% NRS). Extracted starch in CCA was identified as high amylose (71.62 ± 7.18% w/w and 65.85 ± 3.87 % w/w in Trts 1 and 2, respectively). Total monosaccharide content was 1.36 ± 0.11 g/100 g and 1.44 ± 0.09 g/100 g DWB for trts 1 and 2, respectively. Seven monosaccharides were identified. DSC indicated presence of resistant starch. Extracted lipid contents were lower than previous studies this could be due to cellular extraction issues. Total lipid content varies greatly depending on polarity of extraction solvent and technique used. Fatty acids with 13-18 carbons were identified, the most abundant was palmitic acid, linolenic acid and, oleic acid. CCA's ability to grow in several irradiance regimes and

create substantial biomass while still accumulating valuable macronutrients make it a promising source of bioactive compounds.

Chapter 1. Introduction

1.1. Introduction

Microalgae contribute at least a quarter of the biomass of the world's vegetation and is the foundation of the food network by supporting, directly or indirectly, all the species population of the sea. All microalgae species contribute to atmospheric carbon dioxide capturing as part of their photosynthetic activities (Aiken and others, 1992; Jeffrey and Mantoura, 1997), and this helps to counteract green-house gas emission, by removing carbon released to the atmosphere (Sabine and Feely, 2007).

Microalgae contains substantial levels of polysaccharides, polyunsaturated fatty acids, pigments, vitamins, enzymes, bioactive peptides, and minerals (Borowitzka 1988; Ötles and Pire 2001 Cuellar-Bermudez and others, 2015). These bioactive compounds make microalgae a possible vegan source with the added health benefits of the bioactive compounds previously listed. Nutrient content varies based on algae culture type and growth conditions. Very little is known about algal chemical properties in cocultures. Algae is a readily available complete nutrient source meaning it is widely available regardless of growing region, it is a robust crop that can withstand starvation and extreme temperatures, pH and other environmental factors, no viable farmland is required to grow algae, and it sequesters CO_2 from the environment.

Characterization is necessary to understand the components and properties of algae and algal products. There is a need for more research on the compounds present in algae strains and cocultures to encourage their use in food products, and applications such as drug delivery, biosensors, foam-stabilizers, and emulsifiers (Pereira, 2018). Adding microalgal components to food and beverage products can add value to current products by increasing vegan sourced

proteins, lipids, and carbohydrates. There is a growing demand for healthy, tasty, sustainable, low impact, plant-based, high-protein foods. Algae fits this consumer demand completely. Microalgal products need to become more diversified and economically competitive (Spolaore and others, 2006).

This research studied *Chlorella vulgaris* L. (Chlorophyta) /*Leptolyngbya* sp. (Cyanobacteria) co-culture microalgae (CCA). This information is imperative for application of these proteins, carbohydrates, and lipids in the future. This research focused on characterizing the proteins, carbohydrates, starches, and lipids present in *Chlorella vulgaris* L./Cyanobacteria.

1.2. References

Aiken, G. R., D. M. Mcknight, K. A. Thorn, And E. M. Thurman. (1992). Isolation of Hydrophilic Organic Acids from Water Using Nonionic Macroporous Resins. Org. Geochem. 18: 567-573.

Borowitzka, M. A. (1988). Vitamins and fine chemicals from micro-alga. In Microalgal Biotechnology, eds M. A. Borowitzka & L. Y. Borowitzka. Cambridge University Press, Cambridge, UK, p. 153"

Cuellar-Bermudez, S. P., Aguilar-Hernandez, I., Cardenas-Chavez, D. L., Ornelas-Soto, N., Romero-Ogawa, M. A., & Parra-Saldivar, R. (2015). Extraction and purification of high-value metabolites from microalgae: essential lipids, astaxanthin and phycobiliproteins. Microbial biotechnology, 8(2), 190-209.

Jeffrey SW, Llewellyn CA, Barlow RG, Mantoura RFC (1997). Pigment processes in the sea: a selected bibliography. In: Jeffrey SW, Mantoura RFC, Wright SW (eds) Phytoplankton pigments in oceanography. SCOR-UNESCO, Paris, p 167–178

Ötles, S. and Pire, R. 2001. Fatty acid composition of *Chlorella* and Arthospira microalgae species. Journal of AOAC International 84: 1708-1714.

Pereira, L. (2018). Biological and therapeutic properties of the seaweed polysaccharides. International Biology Review, 2(2).

Sabine, C. L., & Feely, R. A. (2007). 3 The Oceanic Sink for Carbon Dioxide. Greenhouse Gas Sinks, 31.

Spolaore, P., Joannis-Cassan, C., Joannis-Cassan, E., Isambert, A., Commercial applications of microalgae – review. J. Biosci. Bioeng. 2006, 101, 87–96.

Chapter 2. Literature Review

2.1. Introduction

Characterizing algal samples called for extensive research into previous studies, and optimal methods to extract, isolate and purify compounds of interest. The following background information was relevant to characterizing the proteins, carbohydrates, starches, lipids and fatty acids present in *Chlorella vulgaris* L./Cyanobacteria *Leptolyngbya.* (CCA).

2.2. Microalgae Species of Interest

2.2.1. Chlorella vulgaris L.

Chlorella is single-cell algae, from phylum Chlorophyta. *Chlorella* uses photosynthesis, to grow rapidly using CO_2, water, light, and minerals to replicate. *Chlorella* is high in protein (Becker, 2007). Health claims associated with *Chlorella* are weight control, cancer prevention, and immune system support. *Chlorella* can be a possible food and energy source because its photosynthetic efficiency can reach 8%, meaning that 8% of the light absorbed by *Chlorella* is preserved as chemical energy, this level of efficiency is comparable to highly efficient crops such as sugar cane (Becker, 1994; Lee and others, 1998; Bewicke and Potter, 2009).

2.2.2. Cyanobacteria Species

Cyanobacteria are single-cell bacteria, they can be free-living or amassed in colonies that form filaments (Sharma and others, 2013; Cyanobacteria, 2017). Cyanobacteria are extremely common in fresh water, where they occur as members of both the plankton and the benthos organism classes. They are also abundant in tide pools, coral reefs, and tidal spray zones. Some cyanobacteria species inhabit the ocean plankton (Sharma and others, 2013; Blue-Green, 2016). On land, cyanobacteria are common in soil down to a depth of 1 m (39 inches) or more.

Cyanobacteria grow on moist surfaces of rocks and trees, where they form cushions or layers (Blue-Green, 2016). Shimura and others 2015 found that terrestrial cyanobacteria *Leptolyngbya* sp. NIES-2104 has the genetic capacity to produce a mycosporine-like amino acid, mycosporine-glycine. Mycosporine-glycine has an antioxidant action, which is thought to contribute to adaptation to terrestrial conditions (Shimura and others, 2015).

Cyanobacteria *Leptolyngbya* are described as filamentous, solitary or coiled into clusters and fine mats (which are sometimes up to macroscopic and several cm in diameter), waved or intensely coiled, iso-polar, thin, fine, and 0.5-3.2 um wide (Komárek,1992). Cyanobacteria *Leptolyngbya* has a straight hair like structure containing tube-shaped cells that do not contain polar gas vacuoles (Anagnostidis and Komárek, 1988; Kim and others, 2015).

2.2.3. Louisiana Native Co-Culture (*Chlorella vulgaris* L./Cyanobacteria *Leptolyngbya* sp.) (CCA)

Louisiana native co-culture of microalgae and cyanobacteria was the sample used for this study. CCA has shown resistance to changing growth parameters like pH and temperature. Silaban, (2013) found that CCA has higher growth rates when compared to monocultured microalgae. CCA shifts species composition from microalgae dominant to cyanobacteria dominant in low light conditions (Bai 2012, Silaban 2013, Barnett, 2015).

Tate and others (2013) found that gene expression of *Chlorella vulgaris* L. in a monoculture compared to that in the co-culture with *Leptolyngbya* sp. was changed. The co-culture was beneficial and efficient because the monoculture eventually fell susceptible to fungal contamination (Tate and others, 2013). This experiment indicated that the co-culture, in which cyanobacteria was 3-7% of the co-culture, was more robust than the monocultures. Culturing a

microalgal polyculture provides increased productivity and reduced contaminants like rotifers, because of differences in cell size and structure of the polyculture (Kent and others, 2015, Corcoran and Boeing, 2012).

This co-culture has been found to be resistant to extreme pH and temperature shifts, and this co-culture has higher growth rates than the microalgae grown in monoculture (Silaban, 2013). Studies have shown co-cultures *Chlorella vulgaris* with *Leptolyngbya* sp. grew 20 times more than *Chlorella vulgaris* in monoculture (Silaban, 2012). The coculture may be able to produce lipids in greater quantities when compared to other species (Silaban, 2012). Augmentation of *Chlorella vulgaris* L. growth by co-culturing with bacteria has been extensively studied (de Bashan and others, 2002; Rasmussen and Nilsson, 2003; Park and others, 2008). Symbiotic relationships between microalgae and cyanobacteria were reported, noting that cyanobacteria could supply nutrient growth factors while decreasing oxygen concentrations aiding in nitrogen fixation (Graham and Wilcox 2000; Silaban 2013). At 80 µmol m^{-2} s^{-1} (low light conditions) the co-culture of *Chlorella*/cyanobacteria shifted from *Chlorella* dominant to cyanobacteria dominant (Barnett and others, 2017). In a study by Barnett in 2015 that investigated the impact of blue, green, red and white light colors on culture growth at 400 µmol m-2 s -1 PAR, it was found that red light caused the highest growth rate (0.41 d^{-1}) and final biomass concentration (913 mg L^{-1}). Bai (2012) found that greater lipid productivity at an irradiance level of 800 µmol m^{-2} s^{-1} with 100% N indicated this was the optimal irradiance level for biomass accumulation of this co-culture. Silaban (2013) found that when Louisiana co-cultures of *Chlorella vulgaris* with *Leptolyngbya* sp. weren't aerated there was an effect of irradiance level on the amount of biomass produced, while in aerated cultures there were no

differences in biomass levels with changes in irradiance level. Mohtashamian (2012) found that the greater the system dilution rate the greater the biomass produced by the Louisiana co-culture.

Microalgal polycultures are used in aquaculture systems as nutrition for fish and crustaceans (Neori, 2011; Dahiya and others, 2012; Kent and others, 2015). There is no published record of polycultures being cultivated for human nutrition. Supplements are on the market currently which combine microalgal species in one multi-nutrient supplement to provide a complete amino acid content and better reflect animal protein.

2.3. Growth Optimization

2.3.1. Light/Irradiance

Previous studies cite that metal halide lights influence the growth of microalgae resulting in increased rates of growth due to photosynthetic efficiency (irradiance~360-400 μmol s^1 m^2) (Benson and Rusch, 2006). Increased light exposure results in greater final algal concentrations. The effects of irradiance and photoperiod on growth rates, fat/water soluble pigments, total protein, and fatty acid content of freshwater green algae have been previously researched by Bouterfas and others (2006), and Barnett and others (2015). The trend in results were that the cell concentration increased with culture growth in continuous light. Fat-soluble pigments were significantly different under different light systems; specifically, chlorophyll-a, which decreased at high irradiance and longer light duration, while β-carotene experienced an inverse trend. Silban (2013) observed that the greatest biomass and neutral lipid production occurred with 2.94 mM nitrogen and irradiance between 400 – 800 μmol m-2 s -1 in a Louisiana co-culture of *Chlorella vulgaris* with *Leptolyngbya* sp. At this same irradiance level, Bai (2012) found that aerated Louisiana co-cultures produced about 7 times more lipid that non aerated cultures with the main fatty acids being C16 and C18 types. Bai (2012) also found that there were no

differences in fatty acids at different irradiance levels in these same co-cultures. White light tended to produce more lipid in co-cultures of *Chlorella vulgaris* with *Leptolyngbya* sp., with the greatest lipid content observed with white light at 1000 µmol m-2 s -1 (Barnett and others, 2015).

2.3.2. Flow Cytometry

Flow cytometry is the measurement of single cells in flowing sample streams (Seckbach, 1999). Flow cytometry is an effective method for screening microalgal cultures (Trask and others, 1982). Cells suspended in water or liquid media are streamed and go through a pulsed beam of light (Yentsch and others, 1983; Olson and others, 1985). Optical detectors assemble scattered light and fluorescent emissions, then use electronics to digitize signals for computer analysis. The light-scatter data provides information about the algal cells, like size, shape, and surface characteristics (Morel, 1991; Marie and others, 2005; Green and others, 2003; Barker and others, 2012). Flow cytometry provides a quantity of event (cells) present in the culture using fluorescence, and forward scatter channel to size algal cells. Aa ratio of algae composition (*Chlorella* to cyanobacteria) can be calculated from gating cells by species. *Chlorella* cells are about 7 µm, while cyanobacteria are about 0.5 um. In flow cytometry, Chlorophyll-a absorbs in the blue -450 nm and red -680 nm spectra range. Fluorescence from chlorophyll-a is usually emitted in the far red -680-720 nm range. Other chlorophylls and carotenoids capture photons and pass them to chlorophyll-a (Cunningham, 1993). Phycoerythrin, phycocyanin, and allophycocyanin absorb blue-green, yellow-orange, and red light, correspondingly. Since algal species contain different amounts and combinations of pigments, it is possible to use a multi-station flow cytometer to collect the fluorescence from each pigment separately. This can be used as an aid to classify phytoplankton from mixed environmental samples (Cunningham, 1993;

Davey and Bell, 1996). Another advantage of using flow cytometry to analyze microalgae is the autofluorescence of naturally occurring intracellular pigments, the pigments can be employed to distinguish between different species or between microalgae and other microorganisms without applying toxic fluorescent probes (Sensen and others, 1993; Hyka and others, 2013).

2.4 Protein in Microalgae

Chlorella vulgaris L. is reported to have 51-58% protein and *Arthospira* platensis (cyanobacteria sp.) is reported to have 46-63% protein DWB (Becker, 2007). There is a consumer trend for high-protein foods, and plant-based proteins. Algal proteins could possibly be used as a source of "green" or vegan proteins and nutraceuticals.

Algal proteins are chiefly enzymatic proteins (Becker, 2007). Protein percent is measured after algae biomass cell wall hydrolysis. Total nitrogen is an estimate of the protein content. In algae there is an overcalculation of the actual protein content because there are other non-protein containing compounds in microalgae such as nucleic acids, amines, glucosamides, and cell wall components (Becker, 1994). The quantity of non-protein nitrogen content varies by species. Ten percent is the general amount considered for non-protein nitrogen content in microalgae (Becker, 1994).

Chlorella vulgaris L. was found to contain the amino acids: Ile: 3.8, Leu: 8.8, Val: 5.5, Lys: 8.4, Phe: 5.0, Tyr: 3.4, Met: 2.2, Cys: 1.4, Try: 2.1, Thr: 4.8, Ala: 7.9, Arg: 6.4, Asp: 9.0, Glu: 11.6, Gly: 5.8, His: 2.0, Pro: 4.8, Ser: 4.1 all reported in g/100g protein (Guedes et al., 2015). The protein from microalgae is considered well-balanced because it contains numerous essential and non-essential amino acids (Becker, 2007). Mohtashamian (2012) observed a range in protein level from 25.5% (DWB) to 49.7 % (DWB) in co-cultures of *Chlorella vulgaris* with

Leptolyngbya sp, depending on the dilution rate and whether lipid was extracted or not before protein analysis. Greater protein content was seen in samples that were not lipid extracted.

Common nutritional quality parameters for protein are protein efficiency ratio (PER) that defined by Becker 2007 as weight gain per unit of protein consumed by the test animal, this is done in short-term feeding trials (Becker, 2007). Biological value (BV) is defined by Becker (2007) as a quantity of nitrogen retained for growth/maintenance (Becker, 2007). Digestibility coefficient (DC) is defined as the digestibility of the tested protein in proportion to the nitrogen that is captured by the test animal by Becker (2007). Net protein utilization (NPU) is the "calculation of BV × DC, which is the quantity of the digestibility of the protein and the biological value of the amino acids absorbed from the food (Becker 2007). Studies (Becker, 2004; Richmond, 2004) found drum dried *Chlorella vulgaris L.* algae samples yielded the subsequent results BV: 76.6, DC: 89.0, NPU: 68.0, and PER: 2.00. Becker (2007) found that algal proteins were comparable to vegetable proteins.

In 1952 Fowden found that in *Chlorella* hydrolysates accounted for 73.9 % of total protein nitrogen was α-amino nitrogen. There is research to suggest that *Chlorella* protein hydrolysate can be used for developing functional foods with immune enhancing activity as shown in mice (Morris and others, 2007). Furthermore, Ursu and others (2014) found that the emulsifying capacity and stability *of Chlorella vulgaris L.* proteins perform as well or outperform commercial ingredients such as sodium caseinate. *Chlorella vulgaris L.* proteins are multifaceted, valuable and competitive in the consumer market.

2.4.1. Phycobiliproteins

Phycobiliproteins are fluorescent photosynthetic complexes according to Glazer in 1994. These proteins can be found in cyanobacteria, red algae and cryptomonads (Glazer, 1989). Phycobiliprotein complexes are grouped into four major categories based on spectral ranges and chromophore make ups. The phycoerythrins and phycoerythrocyanins are absorbed in the blue to green area of 500–565 nm, as well as phycocyanins and allophycocyanins that absorb in the orange (620 nm) to red areas (655 nm) (Bennett and others 1973; Glazer, 1989; Arteni and others, 2009). Phycobiliproteins are water soluble (Glazer, 1989; Barsanti and others, 2008). Phycobiliproteins make up to 40% of the total soluble protein content in algal cells (Chakdar and Pabbi, 2017). Phycobiliproteins participate in efficient energy transfer in photosynthesis (Róman and others, 2002). Phycocyanins found in cyanobacteria species such as *Arthrospira* are used as dietary supplements due to their pharmacological characteristics (Kissoudi, 2017).

Nair and others (2018) found that phycobiliproteins (phycocyanin and phycoerythrin) from the red algae, *Centroceras clavulatum* could be isolated, purified and determined by spectroscopy. They also identified the molecular weight of the phycobiliproteins found as 110 kDa and 250 kDa (by native-polyacrylamide gel electrophoresis) and polypeptide compositions as 17 and 21 kDa (by SDS-PAGE). Patel and others (2005) purified and characterized phycocyanin from cyanobacterial species *(Spirulina* sp., *Phormidium* sp. *and Lyngbya* sp.*)* and the molecular weights of phycocyanin from were 112, 131, and 81 kDa, respectively. They also found two subunits (α and β) of phycocyanin using SDS–PAGE in all cyanobacteria species studied. Each cyanobacterial species displayed a band at 24.4 kDa for the β subunits. The α subunit was displayed at different molecular weights 17 kDa *Spirulina* sp., 19.1 kDa *Phormidium* sp., and 15.2 kDa *Lyngbya* sp. Chen and others (2017) prepared and characterized

food grade phycobiliproteins from *Porphyra haitanensis* then applied these phycobiliproteins in a liposome-meat system. They found that the phycobiliproteins from *Porphyra haitanensis* decreased lipid peroxidation in linoleic acid and the liposome-meat system while providing nutritional value in essential amino acids.

2.5 Carbohydrates in Microalgae

Carbohydrates play many parts in the process of photosynthesis in microalgae and cyanobacteria (Raven and Beardall, 2003). Carbohydrate intermediates, and phosphorylated sugars, affect the photosynthetic carbon reducing process as well as the "photorespiratory carbon oxidation cycle" according to Raven and Beardall in 2003. Carbohydrates are used by the algae as an energy supply and for storage. Algae carbohydrates originate in the chloroplasts of eukaryotes and in the cytosol in prokaryotes (Markou and others, 2012; Safi and others, 2014). Green algae synthesize polysaccharides that are like amylopectin (Markou and others, 2012).

Storage tasked carbohydrates are characteristically starches (amylose and amylopectin). Storage carbohydrates allow algae to survive in dark conditions; however, the amount of time a culture can survive in the dark is species specific (Raven and Beardall, 2003). When microalgae are under stress structurally tasked carbohydrates like soluble cell wall carbohydrates and cellulose are accumulated in the cell wall (Domozych and others, 2012; Ho and others, 2013; Al Abdallah and others, 2016) while starch accumulates in the plastids (Rismani-Yazdi and others, 2011; Ho and others, 2013). Signaling carbohydrates are glycolipids and glycoproteins (Chen and others, 2013; Safi and others, 2014).

It is proposed that *Chlorella's* cell wall consists of polysaccharides attached to phenolic units, like those in lignin (Chen and others, 2017). High temperature, high pH solutions did not

extract polysaccharides (Chen and others, 2017), signifying that attachments with ester bonds were not present (Sui and others, 2012). Rather, cell-wall polysaccharides may be attached with phenolics in ether linkages (Sui and others, 2012). The cell wall polysaccharides of *C. vulgaris* were found to consist of β-(1,3)-glucans, composed of glucose (de Jesus Raposo and others, 2013). The chemical composition of freshwater *C. vulgaris* cell wall components were assayed by Abo-Shady and others (1993), it was found to be 25% hemicellulose, 66.6% rigid wall (alkali insoluble fraction), 30% saccharides, 2.46% proteins, 15% lipids, and 52.54% unknown substances.

Ogawa and others (1999) found that *C. vulgaris* contained 14% uronic acids in polysaccharides. Glucans, found in *Chorella vulgaris* (Nomoto and others, 1983) were found to display several health properties, such as deduction or deterrence of infections and chemoprotective behavior (Bleicher and Mackin 1995). Ortiz-Tena and others (2013) reported the monosaccharide content of *Chorella vulgaris* using HPAED as: glucose: 225.5 mg of monosaccharide/g of dry biomass sample, galactose as 33.7 mg/g, rhamnose as 9 mg/g, mannose as 5 mg/g, ribose as 6.3 mg/g, glucuronic acid as 3.9 mg/g, glucosamine as 4.8 mg/g; xylose as 6.4 mg/g; arabinose as 6.4 mg/g, fucose as none determined and total monosaccharides as 294.6 mg/g. The structure of an aldobiouronic acid isolated from the polysaccharides of various unicellular red algae was evaluated by Geresh and others in 1990. They hydrolyzed and separated extracted polysaccharides then subjected them to thin layer chromatography and HPLC. This total hydrolysis revealed xylose, glucose, galactose, glucuronic acid, rhamnose, arabinose, and 3-*O*-methylpentose, and 4-*O*-methylpentose.

Cyanobacteria synthesize glycogen (Nakamura and others, 2005; Markou and others, 2012). Yim and others (2003) and Trabelsi and others (2009) determined that *Arthospira A.*

platensis (a freshwater cyanobacteria species) contained 5-20% sulfate in polysaccharides, and 7-14.4% uronic acids in polysaccharides (de Jesus Raposo and others, 2013). In cyanobacteria, it is thought that peptide functional groups, protein functional groups and "deoxy-sugars" (rhamnose and fucose) cause polysaccharides to exhibit hydrophobic behavior effecting their emulsifying properties (Flaibani and others, 1989; Shepherd and others, 1995).

2.5.1. Starches in Microalgae

The structure of starch in plants differs from that in bacteria (glycogen) because it presents itself as branched amylopectin (Manners 1991, Gallant et al. 1997, Thompson 2000, Nakamura, 2005). The length of the amylopectin structure is usually 90 ± 0.2 nm among plant species (Jenkins and others, 1993). Another similarity in plant starches is the positioning of the α-1,6-glucosidic linkages being localized in the amylopectin structure so that α-1,4-glucosidic side chains are available to form a double helix (Kainuma and French, 1972; Nakamura, 2005), when the degree of glucose polymerization (DP) of nearby carbohydrate structures reach 10 or more carbons in length (Gidley and Bulpin 1987; Nakamura, 2005). Amylopectin can be catalyzed by three classes of enzymes (starch synthase, starch branching enzymes and starch debranching enzymes), each enzyme is made up of isozymes that effect the amylopectin structure (Nakamura 2002, Ball and Morell 2003; Nakamura, 2005). This differs compared to bacteria starch, glycogen, that can be synthesized by the enzyme glycogen synthase. (Nakamura, 2005). A study by Nakamura in 2015 identified that some cyanobacteria synthesize semi-amylopectin type α-polyglucans in place of glycogen, that is consistent in bacteria. They contribute this change to evolutionary aspects of cyanobacteria's rRNA sequences, and phylogenetic tree.

2.6 Lipids in Microalgae

Eukaryotic algae contain a variety of triacyglycerols (TAGs) (Harwood, 1998). Algae synthesizes lipids most often as membrane lipids that can make up 5-20% of the total dry weight of the algae cell. The TAGs that are present in the greatest quantity in microalgae are usually monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG). The chloroplast membrane lipids are mostly glycosylglycerides Phosphoglycerides are also present inside the cell membrane, cytoplasm, and endoplasmic reticulum of microalgae cells (Guckert and Cooksey, 1990; Harwood, 1998; Pohl and Zurheide, 1979; Wada and Murata, 1998; Al-Hasan and others, 1989; Guschina and Harwood 2006). Many of these TAGs are in hypothesized location is in the thylakoid membranes of microalgae cell's chloroplasts (Al-Hasan and others, 1989; Harwood, 1998). Trigalactosyldiacylglycerol was found to be present in *Chlorella* (Benson and others 1958, Harwood, 1998).

In a study of marine and freshwater algae species, including blue green algae (cyanobacteria sp.) Lee and Loeblich (1971) polar lipids encompass sphingolipids, glycolipids, phospholipids and sterols (Lee and Loeblich, 1971; Al-Hasan and others, 1989). The neutral lipids encompass triglycerides and hydrocarbons. Hydrocarbons can make up to 5% of the total dry weight while the triglycerides contain C_{14}–C_{18} fatty acids that are saturated or mono-unsaturated. (Lee and Loeblich, 1971; Al-Hasan and others, 1989; Aakanksha and others, 2010).

2.6.1 Fatty Acids in Microalgae

Algal lipids are typically made up of fatty acids of the C_{12}-C_{22} array, the most common monounsaturated fatty acid is oleic acid (Matos, 2017). Microalgae gather long-chain fatty acids in the triacylglycerol form of ω-3s specifically α-linoleic acid, eicocosapentanoic acid, and

docosahexanoic acid and ω-6s in the form of linoleic acid, γ-linolenic acid, and arachidonic acid (Armenta and others, 2013; Matos, 2017). These long chain fatty acids have been associated with positive health benefits (Armenta and others, 2013; Matos, 2017). Studies have found that total saturated fatty acids increase, while monounsaturated and polyunsaturated fatty acids decrease when exposed to increasing irradiance and light duration (Benavente-Valdés and others, 2016; Seyfabadi and others 2011).

Cyanobacteria encompasses small quantities of fatty acids, saturated and monounsaturated fatty acids, as well as trace amounts of PUFAs, mostly α-linoleic acid (Lang and others, 2011). In a previous study on irradiance and photoperiod's effect on fatty acid percent in *C. vulgaris*, it was found that the 16:8-h light/dark photoperiod yielded the best results for fatty acids with those being 28.67% saturated fatty acids, 15.15% monounsaturated fatty acids, and 25.58% polyunsaturated fatty acids (Seyfabadi and others 2011).

Petkov and Garcia in 2007 identified the fatty acid composition of *Chlorella* species under photoautotrophic, heterotrophic, nitrogen starvation, and in outdoor photobioreactor conditions, results can be seen in Table 2.1.

Table 2.1. Percentage of fatty acid composition of *Chlorella* species

Percentage of fatty acid composition of *Chlorella* species

Fatty acid	*Chlorella* sp.*	*C. vulgaris*		*C. pyrenoidosa*		
	8000 lx	8000 lx	Outdoor	Photoautotrophic	−N, Photoautotrophic	Heterotrophic
14:0	9 ± 0.5	0.5 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	0.4 ± 0.2	1 ± 0.5
16:0	25 ± 1.5	26 ± 1.5	12 ± 6	22 ± 4	25 ± 2	22 ± 6
$16:1^{7}$	2 ± 0.1	2 ± 0.5	8 ± 3	2 ± 0.5	2.1 ± 0.5	5 ± 2
$16:1^{9}$	–	–	–	1 ± 0.5	0.4 ± 0.2	2 ± 1
16:2	10 ± 0.8	10 ± 1.5	8 ± 3	7 ± 2	9 ± 2	7 ± 3
16:3	9 ± 0.7	2 ± 0.5	12 ± 3	14 ± 4	9 ± 3	8 ± 3
18:0	0.9 ± 0.2	0.3 ± 0.1	0.5 ± 0.4	0.8 ± 0.4	1.1 ± 0.4	4 ± 2
$Z\text{-}18:1^{9}$	5 ± 0.7	16 ± 2	12 ± 4	5 ± 2	8 ± 2.5	6 ± 2
$18:1^{x}$	–	–	–	1.5 ± 0.7	1.4 ± 0.2	4 ± 2
18:2	20 ± 1.2	24 ± 1.5	20 ± 3	18 ± 1.5	27 ± 2	21 ± 4
$18:3^{9,12,15}$	19 ± 0.9	20 ± 2	27 ± 7	27 ± 3	17 ± 2	18 ± 2

Data are mean value and standard deviation of three repetitions.

*Marine species; −N, Nitrogen deprivation.

Table sourced from (Petkov and Garcia 2007)

In a study of *C. vulgaris* lipids cultivated in both organic and inorganic media Murakami and others (1997) found that there were 2.8% neutral lipids in organic media and 1.88% in inorganic media. These authors also found was 8.6% phospholipids in organic media and 6.2% in inorganic media, 5.7% glycolipids in organic and inorganic media, and 1.6% trans-hexadecanoic acid in organic media and 2.3% in inorganic media (Murakami and others, 1997).

2.6.2 Lipid Soluble Pigments

Chlorophylls are green pigments that contain porphyrin and four pyrrole subunits (Bonkovsky and others, 2013). Due to porphyrin's stability, which is caused by its circle-shaped molecular conformation, it can gain or lose electrons. This plays a role in chlorophyll capturing sunlight and turning it into energy (Rowan, 1989). There are three chief chlorophylls, the most prominent being chlorophyll-a. Chlorophyll-a makes photosynthesis possible (Rowan, 1989; Waggoner and Speer, 1999). Chlorophyll-a passes electrons to molecules that ultimately manufacture sugars. Algae, cyanobacteria and any photosynthetic plant contain chlorophyll-a. Chlorophyll-b aids during photosynthesis through absorbing light energy. Chlorophyll-b is more soluble than chlorophyll-a in polar solvents (Rowan, 1989). Chlorophyll-b can only be found in green algae and plants. Chlorophyll-c differs from chlorophyll-b in that it is more unsaturated and doesn't contain an esterified phytol side chain. Chlorophyll-c is found only in photosynthetic Chromista and dinoflagellates (Rowan, 1989; Waggoner and Speer, 1999). Chlorophyll-c pigments are widely distributed among marine and freshwater algae (Rowan, 1989).

Carotenoids are tetraterpenoids (deMan, 1999), they are classified as photosynthetic accessory pigments (Cogdell, 1978) because they transfer their harvested energy to chlorophyll. Carotenoids have a role in microalgae's light collecting. According to Galasso and others (2017) carotenoids make up to 8–14 % of microalgae DWB biomass. Carotenoids can be carotenes or

xanthophylls (Priyadarshani and others, 2012). The carotene carotenoids contain the compounds β-carotene and lycopene. The xanthophyll carotenoids contain the compounds lutein and astaxanthin, (Eonseon, and others, 2003; Fassett and Coombes 2011; Henríquez and others, 2016). Carotenoids synthesized in microalgae are classified as primary and secondary carotenoids (Priyadarshani and others, 2012). Primary carotenoids are crucial to cell life because they aid in both structural and functional parts of cell photosynthesis. The secondary carotenes are only collected after exposure to certain environmentally induced factors (Eonseon, and others, 2003; Henríquez and others, 2016).

2.7. Algae Industry Applications

Macroalgal polysaccharides such as agar, alginates, and carrageenans have been used in several industrial areas for their gelling and thickening properties (Pulz and Gross, 2004). The following genera of microalgae are considered GRAS (Generally Regarded as Safe) by the U.S. Food and Drug Administration and can be consumed as a food source: *Arthospira*, *Chlorella*, *Dunaliella, Haematococcus*, and the oil of *Schizochytrium* (Chacón-Lee and González-Mariño 2010). *Chlorella* is used as an animal feed for larval mollusks and penaeid shrimp (Brennan and Owende, 2010) and was one of the original microalgae species to be commercialized as a food for health (Borowitzka, 2013).

2.7.1. Uses of Algae

Microalgal-based biofuel is a renewable resource; the biofuel has no net emissions of carbon dioxide or sulfur to the atmosphere (Xu and others, 2006). Microalgal biofuels can be produced on land with low agricultural value and low-quality water. Algae can be grown in environments not conducive to growth of terrestrial plants (Tate and others, 2013), using saline

or brackish water or even wastewater (Ferrell and others, 2010). Algae can provide added benefits by removing nitrogen and phosphorous from the wastewater (Li and others, 2008).

2.7.2. Algae as a Food and Nutraceutical

Microalgae has been found to be a source of functional ingredients with positive health effects due to them being high in polyunsaturated fatty acids, polysaccharides, natural pigments, essential minerals, vitamins, enzymes and bioactive peptides (Cuellar-Bermudez and others, 2015). Algae's high protein content also makes it a valuable food component, as it can be an alternative to animal protein for vegetarians and vegans who play an important role in the consumer market. Freshwater algae total protein content can range anywhere between 50-70%. It is the highest protein-rich food in the entire plant kingdom and includes all the essential amino acids making it a complete protein source. Microalgal products need to become more diversified to be economically competitive (Spolaore and others, 2006).

Processes such as distillation, fermentation, or catalytic conversion are also used to obtain fatty acids, glycerol, alcohols (Xu and others, 2006), and other high-value components such as β-carotene, ω-3 fatty acids, and bioplastics (Spolaore and others, 2006) from algae. Large photo-bioreactors mass-produce algal species for market use so that biomass of the desired algae strains and their valuable biochemicals can be produced rapidly. Examples of this are, *Dunaliella* algae species that is rich in carotenoids and therefore is used as an industrial source of β-carotene (de Jesus Raposo and others, 2013), and *Haematococcus* which contains xantophiles specifically, astaxanthin (Levy, 2001). Both substances are in strong demand in the international marketplace as pigments and for well-known health benefits. *Dunaliella* and *Haematoccoccus* algae are used as additives for poultry, crustaceans and fish feeds, to provide bright colors in egg yolks, skin, and fatty tissues due to its pigmenting properties (Sanchez and others, 2008).

Macroalgal polysaccharides such as agar, alginates and carrageenans are used in various fields of industry for their rheological gelling and thickening properties (Pulz and others, 2004). *Arthospira* (Cyanobacteria sp.) has a higher amount of protein by weight than red meat (Fleurence, 1999). *Arthospira platensis* (*Spirulina*) is a complete food supplement and is used to aid in malnutrition in developing countries. *A. platensis* has been added to many commercials food systems like soups, sauces, pastas and snacks but the fishy odor of algae must be overcome to make these products as marketable as possible (Habib, 2008, Cuellar-Bermúdez and others, 2017).

Thrive is the name of an algae oil that is on the market currently created by the algae company Terravia now called Corbion (Amsterdam, Netherlands). Thrive® Algae Oil is an oil made from algae that is marketed to consumers currently. Its health claims are for heart health and they state that one tablespoon provides 13g of monounsaturated fat and that it has 75% less saturated fat than olive oil. The company markets to the "foodie" trend of a common ingredient from an uncommon or novel source, and the "green" trend being that the company claims the oil's processing has decreased greenhouse emissions. The oil is marketed for use in cooking, baking, and salad dressings (Thrive, 2017). There was also research that DHA supplementation from algal oil could reduce serum triglyceride levels and increase low-density lipoprotein cholesterol, also called "bad" cholesterol) and HDL (high-density lipoprotein cholesterol) in persons without coronary heart disease (Bernstein and others 2012).

Algae powders were historically marketed as fish food in the U.S. But, algae-based ingredients offer health and wellness attributes for human food. There is research that algal powders can boost plant protein levels in cheese crackers or smoothies (Brooks and others, 2010), and they may improve the nutritional profile of an ice cream product. *Chlorella vulgaris*,

a green algae species, can be up to 55% protein and this is marketed as a complete vegetable protein (Becker, 2007).

Algae supplements in the form of capsules, tablets, and powders are commercially available currently. Typically, blue green algae supplements are marketed containing *Arthospira*, sometimes in combination with other algae such as *Chorella* or *Aphanizomeron flos aquae* (Nicoletti, 2016).

A *Chlorella* microalgal flour, produced from biomass currently has a patent. It is comprised of lysed cells in the form of a powder, this powder is 40% protein, 20% of triglyceride oil, 10% dietary fiber, 20% carbohydrate and 10% or less moisture by dry weight. The flour is processed by creating micronized microalgal biomass that is emulsified and dried. This flour can be used in the place of historical flours to increase nutritional content and is gluten free (Brooks and others, 2010).

A company called Simris World sells a variety of algal products of these teas comprised of organic traditional herbs and *Dunaliella* algae the product is called “Flower Power Algae Tea” and a product called “Sun Candy Algae Tea” and it is marketed as packed with beta-carotene, with emphasis on its benefits for skin (Hejazi and Wijffels, 2004).

According to the Algae Biomass Organization some U.S. based algal companies are LanzaTech (Skokie, IL), Qualitas Health Inc (Houston, TX), Triton Algae Innovations (San Diego, CA), Earthrise Nutritionals LLC (La Jolla, CA), Sapphire Energy (San Diego, CA), Corbion (Amsterdam, Netherlands), Neste (Espoo, Finland), AstaReal Inc USA (Burlington, NJ**)**, and the National Center for Marine Algae and Microbiota (NCMA) (East Boothbay, Maine) (Algae Biomass Organization, 2019).

Japan, China, Taiwan, India and, Mexico have been growing algae at the large scale since the 1960s (Muller-Feuga 1996; Pulz, and Scheibenbogen, 1998, Borowitzka, 1999). In China, Arthrospira and *Chlorella* species are used to enhance beverages including health drinks, soft drinks, teas, beers and spirits (Liang and others, 2004). The largest producer of algae powder is Hainan Simai (Hainan, China) (Spolaore and others, 2006).

2.8. Conclusion

Microalgae and cyanobacteria were found by previous studies to have various uses across industries and synthesize several valuable compounds, and the studied species grew well under controlled conditions. The biological compounds of interest have been researched, theorized and in some cases proven to aid in their numerous applications, but since algae is such a large plant family there are more compounds to be discovered and characterized. Nutrient content was found to vary greatly due to algae species, growth conditions, extraction method, and protocol. Characterizing nutrients in algae is imperative for application of these compounds in the future.

After reviewing available research, it was decided to pursue commonly used protocols such as the freeze thaw method, sonication and lyophilization for sample preparation. A modification of Bold Basal Medium and 400 W metal halide lights were chosen to cultivate algae. These methods will be further discussed in the following chapter of this dissertation. Overall the compounds in microalgae show great potential for use as food and in food applications.

2.9. References

Aakanksha, S., S., Guruprasad, S., Ramachandra T.V., (2010). Diversity of Lipids in Algae. Paper presented at the Lake 2010: Wetlands, Biodiversity and Climate Change, Bangalore, India. Conference Abstract retrieved from http://www.ces.iisc.ernet.in/energy/lake2010/Theme%201/aakanksha.pdf

Abo-Shady, A. M., Mohamed, Y. A., & Lasheen, T. (1993). Chemical composition of the cell wall in some green algae species. Biologia Plantarum, 35(4), 629-632.

Anagnostidis, K. and Komárek, J. (1988) Modern approach to the classification system of cyanophytes. 3. Oscillatoriales. Arch Hydrobiol Suppl 80, 327–472.

Aiken, G. R., D. M. Mcknight, K. A. Thorn, And E. M. Thurman. (1992). Isolation of Hydrophilic Organic Acids from Water Using Nonionic Macroporous Resins. Org. Geochem. 18: 567-573.

Al Abdallah, Q., Nixon, B. T., & Fortwendel, J. R. (2016). The Enzymatic Conversion of Major Algal and Cyanobacterial Carbohydrates to Bioethanol. Frontiers in Energy Research, 4(36). doi:10.3389/fenrg.2016.00036

Algae Biomass Organization. (2019). Algae Industry Products & Services Directory. available at: https://algaebiomass.org/resource-center/industry-resources/algae-industry-services-directory/#bigelow (accessed May 2019).

Al-Hasan, R. H., Ali, A. M., & Radwan, S. S. (1989). Effects of light and dark incubation on the lipid and fatty acid composition of marine cyanobacteria. Microbiology, 135(4), 865-872.

Armenta, R. E., & Valentine, M. C. (2013). Single-cell oils as a source of omega-3 fatty acids: an overview of recent advances. Journal of the American Oil Chemists' Society, 90(2), 167-182.

Arteni, A. A., Ajlani, G., & Boekema, E. J. (2009). Structural organisation of phycobilisomes from Synechocystis sp. strain PCC6803 and their interaction with the membrane. Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1787(4), 272-279.

Bai, R. (2012). Lipid Production from a Louisiana Native *Chlorella vulgaris*/*Leptolyngbya* sp. Co-culture for Biofuel Applications. Chemical Engineering Commons. LSU Doctoral Dissertation

Ball, S.G. and Morell, M.K. (2003) From bacterial glycogen to starch: understanding the biogenesis of the plant starch granule. Annu. Rev. Plant Biol. 54: 207–233.

Barker, J. P., Cattolico, R. A., & Gatza, E. (2012). Multiparametric Analysis of Microalgae for Biofuels Using Flow Cytometry. White Paper, BD Biosciences.

Barnett, J. Z., Foy, J., Malone, R., Rusch, K. A., & Gutierrez-Wing, M. T. (2017). Impact of light quality on a native Louisiana *Chlorella vulgaris* L./*Leptolyngbya* sp. co-culture. Engineering in Life Sciences, 17(6), 678-685.

Barsanti, L., Coltelli, P., Evangelista, V., Frassanito, A. M., Passarelli, V., Vesentini, N., & Gualtieri, P. (2008). Oddities and curiosities in the algal world. In Algal toxins: nature, occurrence, effect and detection (pp. 353-391). Springer, Dordrecht.

Becker, E. W. (1994). Microalgae: biotechnology and microbiology (Vol. 10). Cambridge University Press.

Becker, E. W. (2007). Micro-algae as a source of protein. Biotechnology advances, 25(2), 207-210.

Benavente-Valdés, J. R., Aguilar, C., Contreras-Esquivel, J. C., Méndez-Zavala, A., & Montañez, J. (2016). Strategies to enhance the production of photosynthetic pigments and lipids in chlorophycae species. Biotechnology Reports, 10, 117–125. http://doi.org/10.1016/j.btre.2016.04.001

Bennett, A. and Bogorad, L. (1973). Complimentary Chromatic Adaptation in a Filamentous Blue-Green Alga. The Journal of Cell Biology, 58, No. 2, 419.

Benson, A. A., R. Wiser, R. A. Ferrari & J. A. Miller, 1958. Photosynthesis of galactolipids. Journal of the American Chemical Society 80: 4740.

Benson, B. C., & Rusch, K. A. (2006). Investigation of the light dynamics and their impact on algal growth rate in a hydraulically integrated serial turbidostat algal reactor (HISTAR). Aquacultural engineering, 35(2), 122-134.

Bernstein, A. M., Ding, E. L., Willett, W. C., & Rimm, E. B. (2012). A meta-analysis shows that docosahexaenoic acid from algal oil reduces serum triglycerides and increases HDL-cholesterol and LDL-cholesterol in persons without coronary heart disease. The Journal of nutrition, 142(1), 99-104.

Bewicke, D., & Potter, B. A. (2009). *Chlorella*: The Emerald Food. Ronin Publishing.

Bleicher P, Mackin W (1995) Betafectin PGG-glucan: a novel carbohydrate immunomodulator with anti-infective properties. J Biotechnol Healthc 2:207–222

Bonkovsky, H. L., Guo, J. T., Hou, W., Li, T., Narang, T., & Thapar, M. (2013). Porphyrin and heme metabolism and the porphyrias. Comprehensive Physiology.

Borowitzka, M. A. (1999). Commercial production of microalgae: ponds, tanks, tubes and fermenters.

Borowitzka, M. A. (2013). High-value products from microalgae—their development and commercialisation. Journal of Applied Phycology, 25(3), 743-756.

Bouterfas R, Belkoura M, Dauta A (2006). The effects of irradiance and photoperiod on the growth rates of three freshwater green algae isolated from a eutrophic lake. Limnetica 25:647–656

Brennan, L., & Owende, P. (2010). Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. Renewable and sustainable energy reviews, 14(2), 557-577.

Brooks, G., Franklin, S., Avila, J., Decker, S. M., Baliu, E., Rakitsky, W., ... & Norris, L. M. (2010). U.S. Patent Application No. 12/684,893.

Chacón-Lee TL, González-Mariño GE (2010) Microalgae for "healthy" foods – possibilities and challenges. Compr Rev Food Sci Food 9(6):655–675

Chakdar, H., & Pabbi, S. (2017). Algal pigments for human health and cosmeceuticals. In Algal Green Chemistry (pp. 171-188). Elsevier.

Chen, X., Wu, M., Yang, Q., & Wang, S. (2017). Preparation, characterization of food grade phycobiliproteins from Porphyra haitanensis and the application in liposome-meat system. LWT, 77, 468-474. doi: https://doi.org/10.1016/j.lwt.2016.12.005

Corcoran AA, Boeing WJ. (2012). Biodiversity increases the productivity and stability of phytoplankton communities. PloS ONE. 7, e49397. pmid:23173059

Cogdell, R. J. (1978). Carotenoids in photosynthesis. Philosophical Transactions of the Royal Society of London. B, Biological Sciences, 284(1002), 569-579. doi:10.1098/rstb.1978.0090

Cuellar-Bermudez, S. P., Aguilar-Hernandez, I., Cardenas-Chavez, D. L., Ornelas-Soto, N., Romero-Ogawa, M. A., & Parra-Saldivar, R. (2015). Extraction and purification of high-value metabolites from microalgae: essential lipids, astaxanthin and phycobiliproteins. Microbial biotechnology, 8(2), 190-209.

Cuellar-Bermúdez, S. P., Barba-Davila, B., Serna-Saldivar, S. O., Parra-Saldivar, R., Rodriguez-Rodriguez, J., Morales-Davila, S., . . . Chuck-Hernández, C. (2017). Deodorization of Arthrospira platensis biomass for further scale-up food applications. Journal of the Science of Food and Agriculture, 97(15), 5123-5130. doi:10.1002/jsfa.8391

Cunningham, A. (1993). Analysis of microalgae and cyanobacteria by flow cytometry. In Flow Cytometry in Microbiology (pp. 131-142). Springer, London.

Cyanobacteria. (2017). Funk & Wagnalls New World Encyclopedia, 1p. 1.

Davey, H. M., & Kell, D. B. (1996). Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses. Microbiological reviews, 60(4), 641-696.

Dahiya A, Todd J, McInnis A. Wastewater treatment integrated with algae production for biofuel. In: Gordon R, Seckbach J, editors. The science of algal fuels: cellular origin, life in extreme habitats and astrobiology. Dordrecht: Springer Netherlands; 2012. pp. 447–466.

de Jesus Raposo, M. F., de Morais, R. M. S. C., & de Morais, A. M. M. B. (2013). Health applications of bioactive compounds from marine microalgae. Life Sciences, 93(15), 479-486. doi: https://doi.org/10.1016/j.lfs.2013.08.002

deMan, J. M., & deMan, J. M. (1999). Color. Principles of Food Chemistry, 229-262.

Domozych, D., Ciancia, M., Fangel, J. U., Mikkelsen, M. D., Ulvskov, P., & Willats, W. G. (2012). The cell walls of green algae: a journey through evolution and diversity. Frontiers in plant science, 3, 82.

Eonseon, J., Polle, J. E. W., & Lee, H. K. S., M. Hyun, and M. Chang. 2003. Xanthophylls in microalgae: from biosynthesis to biotechnological mass production and application. J. Microbiol. Biotechnol, 13(2), 165-174.

Fassett R, Coombes J (2011) Astaxanthin: a potential therapeutic agent in cardiovascular disease. Mar Drugs 9(3):447–465

Ferrell, J., & Sarisky-Reed, V. (2010). National algal biofuels technology roadmap (No. DOE/EE-0332). EERE Publication and Product Library.

Flaibani A, Olsen Y, Painter TJ (1989) Polysaccharides in desert reclamation: compositions of exocellular proteoglycan complexes produced by filamentous blue-green and unicellular green edaphic algae. Carbohydr Res 190(2):235–248

Fleurence, J. (1999). Seaweed proteins: biochemical, nutritional aspects and potential uses. Trends in food science & technology, 10(1), 25-28.

Food and Agricultural Organization/World Health Organization. (1973). Energy and protein requirement: Report of a Joint FAO/WHO ad hoc Expert Committee, vol. 52, FAO.

Fowden, L. (1952). The composition of the bulk proteins of *Chlorella*. Biochemical Journal, 50(3), 355.

Galasso, C., Corinaldesi, C., & Sansone, C. (2017). Carotenoids from Marine Organisms: Biological Functions and Industrial Applications. Antioxidants, 6(4), 96. doi:10.3390/antiox6040096

Gallant, D.J., Bouchet, B. and Baldwin, P.M. (1997) Microscopy of starch: evidence of a new level of granule organization. Carbohydr. Polym. 32: 177–191.

Geresh, S., Dubinsky, O., Arad, S., Christiaen, D., & Glaser, R. (1990). Structure of 3-O- (α-d-glucopyranosyluronic acid)-l-galactopyranose, an aldobiouronic acid isolated from the polysaccharides of various unicellular red algae. Carbohydrate Research, 208, 301-305. doi: https://doi.org/10.1016/0008-6215(90)80116-K

Gidley, M.J. and Bulpin, P.V. (1987) Crystallisation of malto-oligosaccharides as models of the crystalline forms of starch: minimum chain-length requirement for the formation of double helices. Carbohydr. Res. 161: 291–300.

Glazer, A. N. (1994). Phycobiliproteins — a family of valuable, widely used fluorophores. Journal of Applied Phycology, 6(2), 105-112. doi:10.1007/BF02186064

Glazer, A.N., 1989. Light guides. Directional energy transfer in a photosynthetic antenna. J. Biol. Chem. 264:1-4

Graham, L. E., and L. W. Wilcox. 2000. *Algae*. Upper Saddle River: Prentice Hall.

Green, R. E., Sosik, H. M., Olson, R. J., & DuRand, M. D. (2003). Flow cytometric determination of size and complex refractive index for marine particles: comparison with independent and bulk estimates. Applied Optics, 42(3), 526-541.

Guckert, J. B., & Cooksey, K. E. (1990). Triglyceride accumulation and fatty acid profile changes in *Chlorella* (Chlorophyta) during high Ph-induced cell cycle Inhibition 1. Journal of Phycology, 26(1), 72-79.

Guedes, A.C., Sousa-Pinto, I., Malcata, F.X. (2015). Application of protein of microalgae to aquafeed in Handbook of Marine Microalgae Biotechnology Advances. Se-Kwan Kim (ed.). Chp. 8, pp. 93-126.

Guschina, I. A., & Harwood, J. L. (2006). Lipids and lipid metabolism in eukaryotic algae. Progress in Lipid Research, 45(2), 160-186. doi:https://doi.org/10.1016/j.plipres.2006.01.001

Habib, M. A. B. (2008). Review on culture, production and use of Spirulina as food for humans and feeds for domestic animals and fish. FAO Fisheries and Aquaculture Circular. No. 1034. http://www.fao.org/3/i0424e/i0424e00.pdf.

Harwood J.L. (1998) Membrane Lipids in Algae. In: Paul-André S., Norio M. (eds) Lipids in Photosynthesis: Structure, Function and Genetics. Advances in Photosynthesis and Respiration, vol 6. Springer, Dordrecht

Hejazi, M. A., & Wijffels, R. H. (2004). Milking of microalgae. Trends Biotechnol, 22(4), 189-194. doi: 10.1016/j.tibtech.2004.02.009

Henríquez, V., Escobar, C., Galarza, J., & Gimpel, J. (2016). Carotenoids in microalgae Carotenoids in Nature (pp. 219-237): Springer.

Hyka, P., Lickova, S., Přibyl, P., Melzoch, K., & Kovar, K. (2013). Flow cytometry for the development of biotechnological processes with microalgae. Biotechnology advances, 31(1), 2-16.

Jeffrey SW, Llewellyn CA, Barlow RG, Mantoura RFC (1997). Pigment processes in the sea: a selected bibliography. In: Jeffrey SW, Mantoura RFC, Wright SW (eds) Phytoplankton pigments in oceanography. SCOR-UNESCO, Paris, p 167–178

Jenkins, P.J., Cameron, R.E. and Donald, A.M. (1993) A universal feature in the structure of starch granules from different botanical sources. Starch 45: 417–420.

Kainuma, K. and French, D. (1972) Naegeli amylodextrin and its relationships to starch granule structure. II. Role of water in crystallization of B-starch. Biopolymers 11: 2241–2250.

Kent, M., Welladsen, H. M., Mangott, A., & Li, Y. (2015). Nutritional evaluation of Australian microalgae as potential human health supplements. PloS one, 10(2), e0118985.

Kim, J., Choi, W., Jeon, S. , Kim, T. , Park, A. , Kim, J. , Heo, S. , Oh, C. , Shim, W. and Kang, D. (2015), Isolation and characterization of *Leptolyngbya* sp. KIOST-1, a basophilic and euryhaline filamentous cyanobacterium from an open paddle-wheel raceway Arthrospira culture pond in Korea. J Appl Microbiol, 119: 1597-1612. doi:10.1111/jam.12961

Kissoudi, M. Sarakatsianos, I., Samanidou, V. (2018). Isolation and purification of food-grade C-phycocyanin from Arthrospira platensis and its determination in confectionery by HPLC with diode array detection. J Separation Sci. 41(4): 975-981.

Komárek J. (1992): Diversita a moderní klasifikace sinic (Cyanoprocaryota) [Diversity and modern classification of Cyanobacteria (Cyanoprokaryota). - inaugural dissertation, not published

Lang, Imke et al. “Fatty Acid Profiles and Their Distribution Patterns in Microalgae: A Comprehensive Analysis of More than 2000 Strains from the SAG Culture Collection.” BMC Plant Biology 11 (2011): 124. PMC. Web. 16 Feb. 2018.

Lee, R. F., & Loeblich III, A. R. (1971). Distribution of 21: 6 hydrocarbon and its relationship to 22: 6 fatty acid in algae. Phytochemistry, 10(3), 593-602.

Lee, W. C. Y., Lee, W. H., & Rosenbaum, M. (1998). *Chlorella*: McGraw-Hill Education.

Levy, L. W. (2001). U.S. Patent No. 6,191,293. Washington, DC: U.S. Patent and Trademark Office.

Li, Y., Horsman, M., Wu, N., Lan, C. Q., & Dubois-Calero, N. (2008). Biofuels from microalgae. Biotechnology progress, 24(4), 815-820.

Liang S., Liu X., Chen F., Chen Z. (2004) Current microalgal health food R & D activities in China. In: Ang P.O. (eds) Asian Pacific Phycology in the 21st Century: Prospects and Challenges. Developments in Hydrobiology, vol 173. Springer, Dordrecht

Manners, D.J. (1991) Recent developments in our understanding of glycogen structure. Carbohydr. Polym. 16: 37–82.

Marie, D., Simon, N., & Vaulot, D. (2005). Phytoplankton cell counting by flow cytometry. Algal culturing techniques, 1, 253-267.

Markou, G., Angelidaki, I., & Georgakakis, D. (2012). Microalgal carbohydrates: an overview of the factors influencing carbohydrates production, and of main bioconversion technologies for production of biofuels. Applied microbiology and biotechnology, 96(3), 631-645.

Matos, Â. P. (2017). The Impact of Microalgae in Food Science and Technology. Journal of the American Oil Chemists' Society, 94(11), 1333-1350.

Morel, A. (1991). Optics of marine particles and marine optics. In Particle analysis in oceanography (pp. 141-188). Springer, Berlin, Heidelberg.

Morris, H. J., Carrillo, O., Almarales, A., Bermúdez, R. C., Lebeque, Y., Fontaine, R., ... & Beltrán, Y. (2007). Immunostimulant activity of an enzymatic protein hydrolysate from green microalga *Chlorella vulgaris* L. on undernourished mice. Enzyme and Microbial Technology, 40(3), 456-460.

Mohtashamian, M.S. 2012. "The Use of a Mixed *Chlorella* Cyanobacteria Culture as a Protein Source for Aquaculture". LSU Master's Theses.

Muller-Feuga, A. (1996). Marine microalgae. The stakes of research. French Research Institute for the Exploitation of the Sea, Plouzané.

Murakami, C., Takahashi, J., Shimpo, K., Maruyama, T., & Niiya, I. (1997). Lipids and fatty acid compositions of *Chlorella*. Journal of Japan Oil Chemists' Society, 46(4), 423-427.

Nair, D., Krishna, J. G., Panikkar, M. V. N., Nair, B. G., Pai, J. G., & Nair, S. S. (2018). Identification, purification, biochemical and mass spectrometric characterization of novel phycobiliproteins from a marine red alga, Centroceras clavulatum. International Journal of Biological Macromolecules, 114, 679-691. doi: https://doi.org/10.1016/j.ijbiomac.2018.03.153

Nakamura, Y. (2002) Towards a better understanding of the metabolic system for amylopectin biosynthesis in plants: rice endosperm as a model tissue. Plant Cell Physiol. 43: 718–725.

Nakamura, Y., Takahashi, J.-i., Sakurai, A., Inaba, Y., Suzuki, E., Nihei, S., . . . Kurano, N. (2005). Some Cyanobacteria Synthesize Semi-Amylopectin Type α-Polyglucans Instead of Glycogen. Plant and Cell Physiology, 46(3), 539-545. doi:10.1093/pcp/pci045

Neori A. "Green water" microalgae: the leading sector in world aquaculture. J Appl Phycol. 2011; 23: 143–149.

Nicoletti M. (2016). Microalgae Nutraceuticals. Foods (Basel, Switzerland), 5(3), 54. doi:10.3390/foods5030054

Nomoto K, Yokokura T, Satoh H, Mutai M (1983) Anti-tumor effect by oral administration of *Chlorella* extract, PCM-4 by oral admission (article in Japanese). Gan To Kagaku Zasshi 10:781–785

Ogawa, K., Ikeda, Y., & Kondo, S. (1999). A new trisaccharide, α-D-glucopyranuronosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranose from *Chlorella vulgaris* (Vol. 321).

Ogawa, M. A., & Parra-Saldivar, R. (2015). Extraction and purification of high-value metabolites from microalgae: essential lipids, astaxanthin and phycobiliproteins. Microbial biotechnology, 8(2), 190-209.

Olson, R. J., Vaulot, D., & Chisholm, S. W. (1985). Marine phytoplankton distributions measured using shipboard flow cytometry. Deep Sea Research Part A. Oceanographic Research Papers, 32(10), 1273-1280.

Ortiz-Tena, J. G., Rühmann, B., Schieder, D., & Sieber, V. (2016). Revealing the diversity of algal monosaccharides: fast carbohydrate fingerprinting of microalgae using crude biomass and showcasing sugar distribution in *Chlorella vulgaris* L. by biomass fractionation. Algal Research, 17, 227-235.

Park, Y., Je, K.-W., Lee, K., Jung, S.-E., et al., Growth promotion of *Chlorella* ellipsoidea by co-inoculation with Brevundimonas sp. isolated from the microalga. Hydrobiologia 2008, 598, 219-228.

Patel, A., Mishra, S., Pawar, R., & Ghosh, P. K. (2005). Purification and characterization of C-Phycocyanin from cyanobacterial species of marine and freshwater habitat. Protein Expression and Purification, 40(2), 248-255. doi: https://doi.org/10.1016/j.pep.2004.10.028

Petkov, G., & Garcia, G. (2007). Which are fatty acids of the green alga *Chlorella*. Biochemical Systematics and Ecology, 35(5), 281-285.

Pohl, P. and Zurheide, F. (1979b) Control of fatty acid and lipid formation in Baltic marine algae by environmental factors. In Advances in the Biochemistry and Physiology of Plant Lipids (Appelqvist, L.A. and Liljenberg, C., eds). Amsterdam: Elsevier, pp. 427–432.

Priyadarshani I, Biswajit R (2012) Commercial and industrial applications of micro algae – a review. J Algal Biomass Utln 3(4):89–100

Pulz, O., & Scheibenbogen, K. (1998). Photobioreactors: design and performance with respect to light energy input. In Bioprocess and algae reactor technology, apoptosis (pp. 123-152). Springer, Berlin, Heidelberg.

Pulz, O., & Gross, W. (2004). Valuable products from biotechnology of microalgae. Applied microbiology and biotechnology, 65(6), 635-648.

Rasmussen, U., Nilsson, M., 2003. Cyanobacterial Diversity and Specificity in Plant Symbioses.Cyanobacteria in Symbiosis, in: Rai, A.N., Bergman, B., Rasmussen, U. (Eds.). Springer Netherlands, pp. 313-328.

Raven J.A., Beardall J. (2003) Carbohydrate Metabolism and Respiration in Algae. In: Larkum A.W.D., Douglas S.E., Raven J.A. (eds) Photosynthesis in Algae. Advances in Photosynthesis and Respiration, vol 14. Springer, Dordrecht

Rismani-Yazdi, H., Haznedaroglu, B. Z., Bibby, K., & Peccia, J. (2011). Transcriptome sequencing and annotation of the microalgae Dunaliella tertiolecta: pathway description and gene discovery for production of next-generation biofuels. BMC genomics, 12(1), 148.

Rowan, K. S. (1989). Photosynthetic pigments of algae: CUP Archive.

Sabine, C. L., & Feely, R. A. (2007). 3 The Oceanic Sink for Carbon Dioxide. Greenhouse Gas Sinks, 31.

Safi, C., Zebib, B., Merah, O., Pontalier, P.-Y., & Vaca-Garcia, C. (2014). Morphology, composition, production, processing and applications of *Chlorella vulgaris*: A review. Renewable and Sustainable Energy Reviews, 35, 265-278. doi: https://doi.org/10.1016/j.rser.2014.04.007

Seckbach, J. (1999). Cellular origin, life in extreme habitats and astrobiology.

Sensen, C. W., Heimann, K., & Melkonian, M. (1993). The production of clonal and axenic cultures of microalgae using fluorescence-activated cell sorting. European Journal of Phycology, 28(2), 93-97.

Seo, Y. C., Choi, W. S., Park, J. H., Park, J. O., Jung, K.-H., & Lee, H. Y. (2013). Stable Isolation of Phycocyanin from Spirulina platensis Associated with High-Pressure Extraction Process. International Journal of Molecular Sciences, 14(1), 1778-1787.

Seyfabadi, Z. R., Zahra Amini Khoeyi. (2011). Protein, fatty acid, and pigment content of *Chlorella vulgaris* L. under different light regimes. Journal of Applied Phycology, 23(4), 721-726. doi:10.1007/s10811-010-9569-8

Sharma, N. K., Rai, A. K., & Stal, L. J. (2013). Cyanobacteria: an economic perspective. John Wiley & Sons.

Shepherd R, Rockey J, Sutherland IW, Roller S (1995) Novel bioemulsifiers from microorganisms for use in foods. J Biotechnol 40(3):207–217

Shimura, Y., Hirose, Y., Misawa, N., Osana, Y., Katoh, H., Yamaguchi, H., & Kawachi, M. (2015). Comparison of the terrestrial cyanobacterium *Leptolyngbya* sp. NIES-2104 and the freshwater *Leptolyngbya* boryana PCC 6306 genomes. DNA Research, 22(6), 403-412.

Silaban, A. G. (2013). Growth rate and productivity of a Louisiana native microalgae/cyanobacteria co-culture: Feasibility for use in industrial biotechnology applications. LSU Master's Thesis Commons. pp. 202.

Spolaore, P., Joannis-Cassan, C., Joannis-Cassan, E., Isambert, A., Commercial applications of microalgae – review. J. Biosci. Bioeng. 2006, 101, 87–96.

Sui, Z., Gizaw, Y., & BeMiller, J. N. (2012). Extraction of polysaccharides from a species of *Chlorella*. Carbohydrate Polymers, 90(1), 1-7. doi: http://dx.doi.org/10.1016/j.carbpol.2012.03.062

Tate, John Joseph, "Differential Gene Expression in a Louisiana Strain of Microalgae" (2012). LSU Master's Theses. 3945. https://digitalcommons.lsu.edu/gradschool_theses/3945

Tate, J. J., Gutierrez-Wing, M. T., Rusch, K. A., & Benton, M. G. (2013). Gene expression analysis of a Louisiana native *Chlorella vulgaris* L. (Chlorophyta)/*Leptolyngbya* sp. (Cyanobacteria) co-culture using suppression subtractive hybridization. Engineering in Life Sciences, 13(2), 185-193. doi:10.1002/elsc.201200063

Thompson, D.B. (2000) On the non-random nature of amylopectin branching. Carbohydr. Polym. 43: 223–239

Thrive Algae. (2017). Thrive Algae Oil. Visited 4/16/17. http://www.thrivealgae.com/resources/

Trabelsi L, M'sakni NH, Ouada HB, Bacha H, Roudesli S (2009) Partial characterization of extracellular polysaccharides produced by cyanobacterium Arthrospira platensis. Biotechnol Bioprocess Eng 14:27–31

Trask, B. J., Van den Engh, G. J., & Elgershuizen, J. H. B. W. (1982). Analysis of phytoplankton by flow cytometry. Cytometry: The Journal of the International Society for Analytical Cytology, 2(4), 258-264.

Ursu, A. V., Marcati, A., Sayd, T., Sante-Lhoutellier, V., Djelveh, G., & Michaud, P. (2014). Extraction, fractionation and functional properties of proteins from the microalgae *Chlorella vulgaris*. Bioresource technology, 157, 134-139.

Wada, H., & Murata, N. (1998). Membrane lipids in cyanobacteria. In Lipids in photosynthesis: structure, function and genetics (pp. 65-81). Springer, Dordrecht.

Waggoner, B., & Speer, B. R. (1999). Photosynthetic Pigments. Website: http://www. ucmp. berkeley. edu/glossary/gloss3/pigments. html.

Xu, H., Miao, X., Wu, Q., High quality biodiesel production from a microalga *Chlorella* protothecoides by heterotrphic growth in fermenters. (2006). J. Biotech. 126, 499–507

Yentsch, C. M., Horan, P. K., Muirhead, K., Dortch, Q., Haugen, E., Legendre, L., ... & Spinrad, R. W. (1983). Flow cytometry and cell sorting: A technique for analysis and sorting of aquatic particles1. Limnology and Oceanography, 28(6), 1275-1280.

Yim, J. H., Kim, S. J., Ahn, S. H., & Lee, H. K. (2003). Optimal conditions for the production of sulfated polysaccharide by marine microalga Gyrodinium impudicum strain KG03. Biomolecular engineering, 20(4-6), 273-280.

Yu, M. H. and Glazer, N. A., Cyanobacterial Phycobilisomes. Role of the Linker Polypeptides in the Assembly of Phycocyanin. (1982). The Journal of Biological Chemistry, 257, No. 7, 3429.

Chapter 3. Louisiana Native Co-Culture of Microalgae (*Chlorella Vulgaris* L.) and Cyanobacteria (*Leptolyngbya* sp.) Cultivation

3.1. Introduction

Louisiana native co-culture of microalgae (*Chlorella vulgaris* L.) and cyanobacteria (*Leptolyngbya* sp.) (CCA) was provided by Dr. Gutierrez-Wing in the Aquatic Germplasm and Genetic Resources center of the School of Renewable Natural Resources, LSU Ag Center. To increase cyanobacteria in a culture it was starved for nitrogen, by feeding CO_2 directly, mixing mechanically (Pulz, 2001; Zhang, 2015). The CCA was cultivated using the growth parameters: scalar irradiance, pH, temperature, growth media, and aeration. The growth parameters are discussed in the subsequent sections. Two irradiance treatments were applied to CCA in this study. The objective of this study was to cultivate 6 CCA cultures, 3 cultures were exposed to average scalar irradiance (ASI) 1041 ± 269.18 μmol m^{-2} s^{-1} PAR and will be referred to as Treatment 1 and the other 3 cultures were exposed to ASI 430 ± 96.03 μmol m^{-2} s^{-1} PAR and will be referred to as a Treatment 2 . This study's aim was not to optimize CCA growth, the focus was creating biomass to compare the previously stated treatments.

3.2. Methods for Growing Algae Co-Cultures

Algae was grown in batch systems. In a batch system culture cells were grown in a specific volume of nutrient medium under environmental conditions, no dead cells were removed, and no nutrients were added. Large 120-gallon tanks were used, under 400 W metal halide lights. Cultures were grown in Bold 1NV Medium which is described later in section 3.2.6. (Bold, 1949; Brown and Bold, 1964; Starr and Zeikus, 1993; UTEX, 2016). Cultures were continuously aerated at 45 liters per minute (LPM) using ambient air. Carbon dioxide was injected as needed to provide a carbon source and control the culture pH at around 7.0.

Dechlorinated tap water was used in the culture, and 19 L of inoculum at 25°C. Optical density, pH, temperature and aeration were monitored daily and, flow cytometry was used to determine the ratio of algae species present in the co-culture every 7 days.

Prior to inoculation, the following preparation protocol was used: the tank was cleaned with 6% sodium hypochlorite, the tank was filled with a 50ppm sodium hypochlorite-tap water solution. This solution was aerated for 72 h for disinfection of the water. Sodium thiosulfate (50 ppm) was added to the water with 4 h aeration initially, to remove chlorine. Chlorine was measured, with indicator testing strips. If the water did not read 0 ppm for chlorine at this time, sodium thiosulfate was added in 100 mL aliquots and measured, until chlorine was at 0 ppm. Bold 1NV Medium nutrients (section 3.2.6.) were added and allowed to equilibrate 72 h, inoculum (section 3.2.8.) was then added, with the previously stated parameters measured daily.

Algae was harvested during stationary phase which is determined as on the first day of decrease in optical density, using a semi-continuous flow centrifuge at 2L/min. All cultures were frozen after harvest and stored at 4°C short-term (2 months) or at -20˚C long-term (3-12 months).

3.2.1. Flow Cytometry

3.2.1.1. Sample Preparation

Samples were taken directly from the cultures and sonicated for 3 min at 40% AMP, pulse 20s on/10s off, on ice (Hyka and others, 2013; Gutierrez-Wing, unpublished work). Samples were run on the flow cytometer and if the initial cell count was higher than 10,000/µL. diluted 10x with deionized water.

3.2.1.2. System Conditions

In this study cell concentrations (cells μL^{-1}) were measured by flow cytometry with a BD Accuri™ C6 Flow Cytometer. CCA cells were excited by blue (488nm) and red laser (640nm). Fluorescence from CCA was captured in filters FL3 (excitation wavelength of 488 nm and emission of > 670 LP) and FL4 (excitation wavelength of 640 nm and emission of 675 ± 12.5). Optical channels of forward scatter channel (FSC) 0 ±15˚used to detect cell size and side scatter channel (SSC) that detects 90 ±15˚with FL3 and FL4 were used to calculate the abundance of *Chlorella* and cyanobacteria in CCA. FL3 was used to identify chlorophyll-a and FL4 to identify phycocyanin. The filters were chosen based on previous studies (Barnett and others, 2017). Data was analyzed using BD Accuri™ C Flow Plus software. An aliquot of the culture was analyzed every seven days to ensure culture growth and validate culture species ratio.

The flow cytometry protocol began by checking fluid levels in all bottles, the sample injection port (SIP) was then cleaned by performing a backflush with sheath fluid 2-3 times to remove clogs and residue at the base of SIP, dripping sheath fluid was seen coming from the SIP. The startup protocol was run as follows: an Eppendorf tube with 2 mL of de-ionized water was placed on the SIP then the cursor was moved to any empty data well in the C Flow software, the time limit was set for two minutes, nominal flow rate was set to fast (66 ul/min), and the system was run, deionized water was run first, until number of events was <10/ul. An Eppendorf tube containing 1 mL of well-mixed culture was then placed on the SIP and ran for 2 mins at a nominal flow rate: slow (14 ul/min).

The BD Accuri C6 flow cytometer cyanobacteria cell detection range was $10^1 – 10^3$ RFU (relative fluorescence units), *Chlorella* cell range detection limits were 10^4-10^6 RFU. The

relative concentration of microalgal and cyanobacterial cells was determined by their autofluorescence due to chlorophyll and phycocyanin.

3.2.2. Scalar Irradiance

Treatment 1 was a culture exposed to average scalar irradiance (ASI) of 1041 ± 269.18 μmol m^{-2} s^{-1} PAR. This irradiance was obtained by suspending metal halide (400 W) lamps 10" above the culture's surface. Treatment 2 was a culture exposed to ASI of 430 ± 96.03 μmol m^{-2} s^{-1} PAR. This irradiance was obtained by suspending metal halide (400 W) lamps 25" above the cultures surface, diffusing the light. Previous studies showed that with reduced light a decrease in *Chlorella* growth was observed, allowing cyanobacteria ratio to increase (Bai, 2012; Silaban, 2012; Barnett and others, 2015).

A light sensing logger LI-COR 1400 paired with an LI-193 Underwater Spherical Quantum Sensor was used to evaluate light initial scalar irradiance. Distribution of light in the tanks varied so a weighted average was taken to determine the initial light intensity. The initial scalar irradiance was measured in tap water. Each tank was divided into 3 depths (1, 14 and 27" from surface of the water) and 3 concentric rings. To determine the concentric rings, 4 measurements were taken at each depth, one at the center, 8, 16 and 24 inches away from the center. Scalar irradiance for each ring was determined by average the light intensity from two points of the ring multiplied by the surface area of the respected ring. For example, for the center ring the average scalar irradiance would be average light intensity at the center and 8" multiplied by the area. For the next ring the average scalar irradiance would be the average light intensity at 8 and 16" multiplied by the surface area of the ring. The summation of each scalar irradiance determined the weighted sum at a specific depth. The average of all the weighted sums over all

the depths determined the initial scalar irradiance of the tank (Barnett and others, 2015; Benson and Rusch, 2006).

3.2.3. Optical Density

Samples were taken directly from the culture, then agitated to suspend cells in culture sample before reading absorbance. If the initial absorbance at 620 nm was higher than 2.000 the sample was diluted 10x with deionized water.

Optical density served as a gauge of algal growth and was measured daily. Optical density (OD_{620}) was obtained by measuring absorbance at 620 nm in a spectrophotometer (Thermo Genesys® 10 UV UV/VIS). Optical density provides an approximation of culture cell concentration. When analyzed for absorbance the culture gets darker as it grows causing absorbance to increase. Culture growth curves were plotted as absorbance against time (in days). CCA growth phase and harvest day were determined by observing a decrease in optical density of CCA on the culture growth curves as indication of onset stationary phase (around day 32).

3.2.4. pH

Values were measured on a standard calibrated pH meter (Mettler Toledo FEP20) daily. The culture was dosed with CO_2 when the pH was above 9 to increase growth. The optimal pH for CCA growth is achieved maintaining a culture pH of 7.0-8.0 (Gutierrez-Wing and others, unpublished work).

3.2.5. Temperature

The culture temperature was monitored daily with glass alcohol thermometer that was graduated 10 - 110°C (Miniscience). The optimal temperature based on previous studies on this co-culture was room temperature (25 ± 2˚C).

3.2.6. Bold 1NV Culture Formula

The Bold 1NV Medium recipe is a modified version of Bolds recipe. It modified for xenic freshwater cultures. The recipe was a UTEX formula (Bold, 1949; Brown and Bold, 1964; Starr and Zeikus, 1993; UTEX, 2016).

To make 200 mL of Vitamin B_{12} solution, 200 mL of HEPES buffer was prepared (50 mM or 2.4g in 200mL DI water), the HEPES buffer pH was adjusted to 7.8, then 0.027g of vitamin B_{12} cyanocobalamin was added and fully dissolved. This solution was sterilized by 0.45 µm Millipore filter and stored in a dark freezer. To make 200mL of Biotin solution, 200 mL of HEPES buffer was prepared as previously stated, then 0.005g of Biotin was added and fully dissolved. The solution was sterilized and stored a previously stated. To make 50mL of Thiamine solution, 50 mL of HEPES buffer was prepared, then 0.067g of Thiamine was added and fully dissolved. This solution was sterilized and stored as previously stated (Bold, 1949; Brown and Bold, 1964; Starr and Zeikus, 1993; UTEX, 2016). The vitamin solution consisted of were 1mL of Vitamin B_{12} solution, 1mL of Biotin solution, and 1mL of Thiamine vitamin solution. These solutions were stored in the refrigerator at 4˚C.

To make 1 L of P-IV Metal solution: 0.75g of $Na_2EDTA•2H_2O$, 0.097 g of $FeCl_3•6H_2O$, 0.041 g of $MnCl_2•4H_2O$, 0.005 g of $ZnCl_2$, 0.002 g of $CoCl_2•6H_2O$, and 0.004 g of $Na_2MoO4•2H_2O$ was added to approximately 950 mL of DI water, and stirred continuously then brought up to 1 L volume. Medium was covered and autoclaved, then cooled to add vitamins.

To make 1 L of Bold 1NV Media the recipe was as follows: 10mL of 2.94mM $NaNO_3$, 10 mL of 0.17mM $CaCl_2·2H_2O$, 10mL of 0.3mM $MgSO_4·7H_2O$, 10mL of 0.43mM K_2HPO_4, 10mL of 1.29mM KH_2PO_4, 10mL of .043mM NaCl, and 6mL of P-IV Metal Solution was added to approximately 900 mL of DI water, each of the components were added in the order specified

(except vitamins) while stirring continuously, the volume was then brought up to 1 L volume. These solutions were stored in the refrigerator at 4˚C.

3.2.7. Aeration

Aeration was provided from two air pumps (Danner Manufacturing, Inc. Pondmaster AP100, #04580) connected to tanks by silicone tubing and a distribution manifold submerged inside the culture. Air flow was measured by water displacement. For the purposes of this study, water displacement was used as a measure of aeration by being a measure of volume. A cylinder of known volume was submerged in the culture water upside down, the air hose nozzle was placed in the cylinder causing air to displace the culture in the cylinder, a timer was running concurrently as the water was displaced by the air. This process was timed and provided a rate of volume of air/second. Knowing the amount of time needed to displace the culture and the volume of the cylinder allows the aeration to be quantified in liters per second (Gutierrez-Wing, unpublished work).

Aeration in this study served as a physical source of continuous mixing of the cocultures. The air bubbles allowed algae cells to scatter, and kept the cells suspended in culture. This allowed cells to cycle to higher/lower irradiance in the tank. The air pump connected to tank 1 had a greater capacity to aerate cultures, when compared to tank 2, as the LPM (liters per minute) is 42.20 ± 3.60 and 39.92 ± 0.90 LPM respectively. There was no significant difference between aeration pumps of tank 1 and tank 2 at $p<.05$ ($p= 0.34$).

3.2.8. Inoculum Cultivation

Inoculum was grown from CCA that was provided by Dr. Gutierrez-Wing in the Aquatic Germplasm and Genetic Resources center of the School of Renewable Natural Resources, LSU

Ag Center. Inoculum was received in small 250mL cultures, it was grown in 19 L batches under LED lamps, in Bold 1NV Medium, at ~45 LPM using continuous ambient air, the pH was maintained at pH 7 – 9 with CO_2, the temperature was maintained at room temperature (~25 ± 2°C). Optical density and flow cytometry were observed to identify onset of stationary phase in the inoculum culture.

3.2.9. Chlorination

Chlorine level was analyzed by chlorine testing strips with 0-200 ppm testing limits (Precision Laboratories, Cottonwood, AZ). This testing was only necessary after adding tap water and 6% sodium hypochlorite solution (bleach) to the 120-gallon tank to start a culture and after cleaning the tanks between cultures. Air flowed into the chlorinated water for several hours and effectively evaporated chlorine. Sodium thiosulfate was added to ensure that there was no chlorine present prior to the start of the cultures.

3.2.10. Experimental Design

The experiment was conducted in a completely randomized design (CRD), with no blocks. Six tanks were chosen at random to contain one of the two treatments, Treatment 1 – cultures exposed to ASI of 1041 ± 269.18 μmol m^{-2} s^{-1} PAR and Treatment 2– cultures exposed to ASI 430 ± 96.03 μmol m^{-2} s^{-1} PAR. The co-culture species ratio (*Chlorella*: Cyanobacteria) was the response of the treatments. The experimental and sampling units were the algae tanks. An illustration of the experimental design can be seen in Figure 3.1. The different analyses are the dependent variables that were compared between treatment levels.

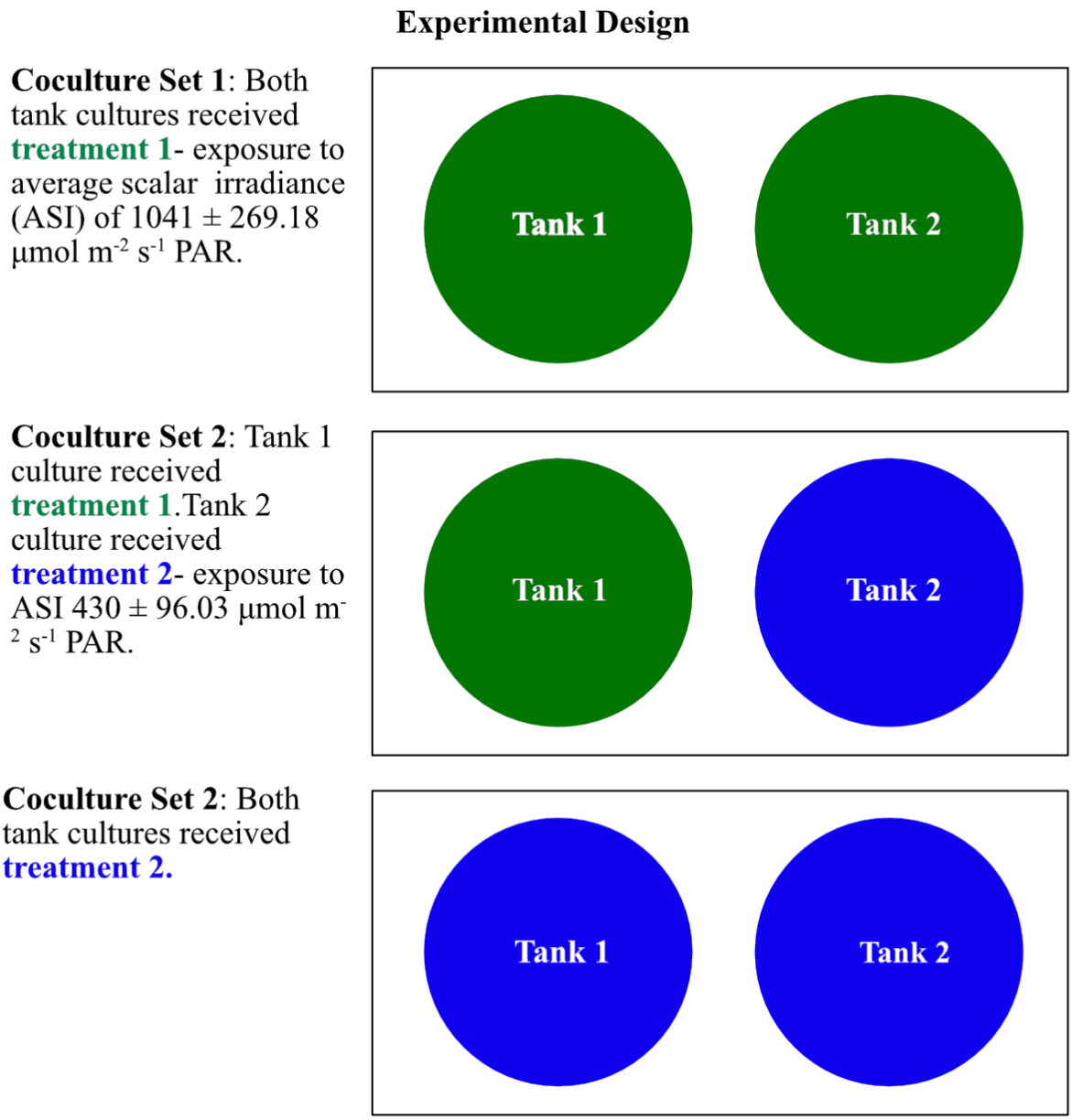


Figure 3.1. Experimental design of algae cultivation

3.2.11. Statistics

Statistical analysis was performed using two-sample t-test (GraphPad, QuickCalc https://www.graphpad.com/quickcalcs/). The two-sample t-test was performed at 95% confidence and compared the average means of each dependent variable (pH, temperature, and aeration) by treatment.

3.3. Results and Discussion

Biomass wet weight was measured post-harvest from each of the six tanks. They were found to be 0.76 ± 0.20 g/L or 760 ±200 g/m^3 for treatment 1, and 0.71 ± 0.13 g/L or 710±130 g/m^3 for treatment 2. There was no significant difference in biomass weight between treatments at 95% confidence ($p = 0.71$). The high wet biomass weight is a relative indicator that CCA responded positively to the treatments applied. Barnett (2015) found that the average volumetric and areal productivity for the last tank in a continuous flow hydraulically integrated serial turbidostat algal reactor (HISTAR) system was 25.0 g m^{-3} d^{-1} and 13.3 g m^{-3} d^{-1} for CCA cultures exposed to ASI 454 μmol m^{-2} s^{-1}. Silaban (2013) grew CCA at ASI 550 μmol m-2 s-1 and found 27.0 g m^{-3} d^{-1} for flow rate 1 L/min.

3.3.1. Flow Cytometry

The CFlow Plus program provided a table with a quantification of events, this table displayed cell concentration verification (Table 3.1). The tables showed results of cell counts, in a sample volume of about 26.6 μL for each tank that grown. The cultures cells μL^{-1} increased as the number of days the culture was grown increased.

From the flow cytometer (Figure 3.2 and Table 3.1) it can be determined that *Chlorella vulgaris* L. was the dominant species in CCA, when compared to cyanobacteria *Leptolyngbya*. This was the expected response from the irradiance levels each treatment was exposed to. *Chlorella* was the dominant species present in CCA from starter culture to harvest. Figure 3.2. displays the plot of flow cytometry when comparing the two treatment levels of species at different ratios. Using the proper channels FL4-A, and FL3-A determined cyanobacteria (Cya)and *Chlorella* (Chl). Two distinct species can be seen in the flow cytometry spectra in Figure 3.2. FSC-A vs. FL3-H was used to identify the two algae species by sorting by size and pigment respectively. The top

image (Figure 3.2.) shows treatment 1 in the top images, a large *Chlorella* population was enclosed by P7 on the left and M4 on the right. The treatment 2 bottom images show a slightly increased Cyanobacteria population enclosed in P4 on the left and M4 on the right.

Treatment 1: culture exposed to average scalar irradiance (ASI) of 1041 ± 269.18 µmol m^{-2} s^{-1} PAR

P7 (left image), M4 (right image) *Chlorella vulgaris* L.
P8 (left image), M3 (right image) Cyanobacteria *Leptolyngbya* sp.

Treatment 2: culture exposed to ASI 430 ± 96.03 µmol m^{-2} s^{-1} PAR

P5 (left image), M1 (right image) *Chlorella vulgaris* L.
P4 (left image), M4 (right image) Cyanobacteria *Leptolyngbya* sp.

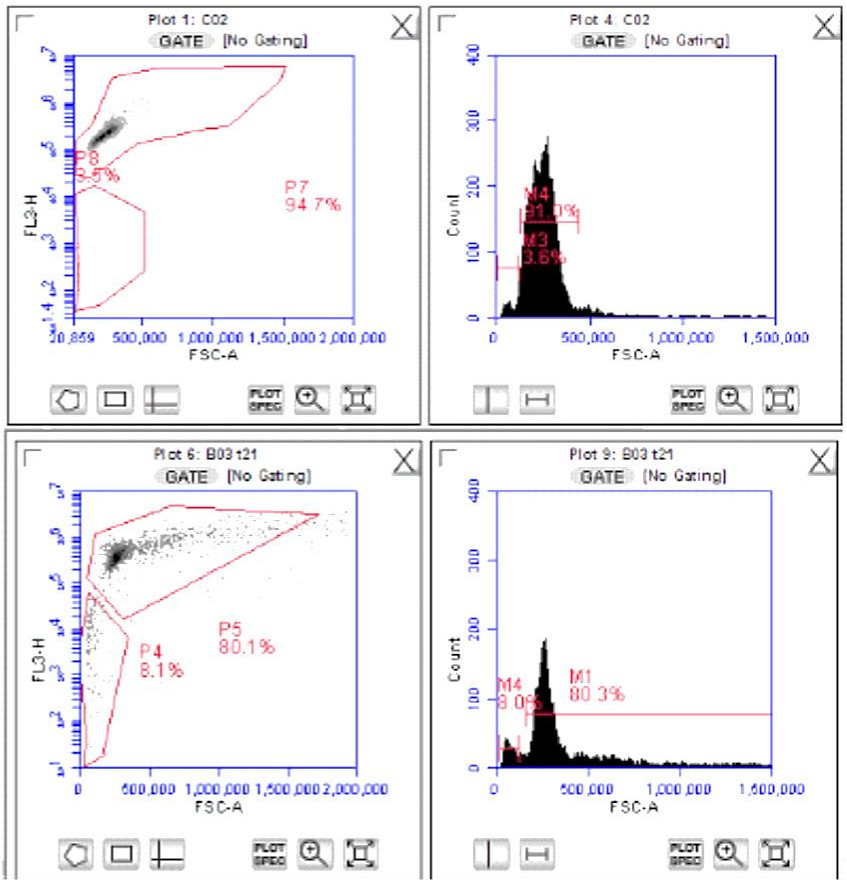


Figure 3.2. Flow cytometry profiles for CCA

Species ratio in the CCA culture was determined from flow cytometry (Table 3.1.), using gating to select the CCA cells from debris, then each species cells respectively based on size and fluorescence. Trt (treatment) 1 compared to trt 2 had significantly greater Chl (*Chlorella*) and significantly less Cya (Cyanobacteria). The opposite was true for trt 2. The species ratio is a response to the applied treatments to the cultures. The irradiance levels controlled the species growth as expected meaning the higher irradiance trt 1 produced an average culture ratio of 97.47 ± 1.29% Chl, and 2.84 ± 1.27% Cya. Trt 2 yielded an average culture ratio of 89.85 ± 1.17

Chl, and 10.64 ± 1.97 Cya. Trt 1 contained significantly more Chl at 95% confidence than trt 2 (p=0.001). Cya in trt 2 was significantly higher at 95% confidence when compared to trt 1.

Table 3.1. CCA Species Ratio Comparison

Tank 1 Culture Set 1

Growth Day	Trt	Tank	Chl%	Cya%	cells/uL
2	1	1	96.24	3.76	630
9	1	1	97.95	2.09	1538
16	1	1	96.90	3.10	2539
23	1	1	98.38	1.62	3559

Tank 2 Culture Set 1

Growth Day	Trt	Tank	Chl%	Cya%	cells/uL
2	1	2	96.34	1.75	846
9	1	2	97.51	2.73	2028
16	1	2	97.65	2.64	3054
23	1	2	98.04	2.73	4065

Tank 1 Culture Set 2

Growth Day	Trt	Tank	Chl%	Cya%	cells/uL
1	1	3	98.25	1.75	837
6	1	3	97.27	2.73	1297
15	1	3	97.43	2.64	2726
21	1	3	97.34	2.73	2817
32	1	3	96.00	4.16	2686

Tank 2 Culture Set 2

Growth Day	Trt	Tank	Chl%	Cya%	cells/uL
1	2	4	97.05	3.04	515
6	2	4	96.26	3.89	949
15	2	4	97.02	3.01	1024
21	2	4	96.85	3.26	2015
32	2	4	88.62	12.84	1414

Tank 1 Culture Set 3

Growth Day	Trt	Tank	Chl%	Cya%	cells/uL
1	2	5	92.42	8.20	434
6	2	5	98.07	1.96	581
13	2	5	95.78	4.40	894
21	2	5	91.94	8.77	1462
27	2	5	89.96	10.04	1683

Tank 2 Culture Set 3

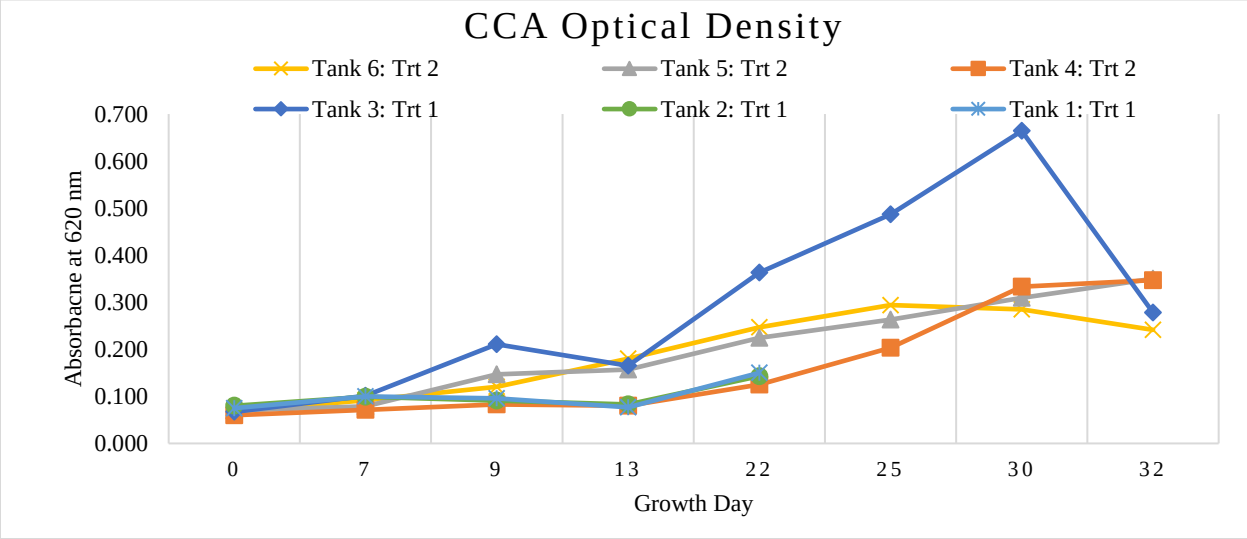
Growth Day	Trt	Tank	Chl%	Cya%	cells/uL
1	2	6	87.90	12.10	157
6	2	6	96.26	3.59	375
13	2	6	95.78	4.4	474
21	2	6	91.82	8.18	699
27	2	6	90.96	9.04	1365

Trt means treatment. Treatment 1 average scalar irradiance of 1041 ± 269.18 μmol m-2 s-1 PAR Treatment 2 ASI of 430 ± 96.03 μmol m-2 s-1 PAR.

3.3.2. Optical Density

An optical density growth curve of absorbance at 620 nm vs. growth day was expressed in Figure 3.3. Cocultures 1-2 were harvested at day 22 while in the exponential growth phase by research error. The subsequent cocultures were harvested day 32 of growing after reaching exponential or stationary phase, stationary phase is represented by a decrease in absorbance at 620 nm. Table 3.4. displays absorbance values of Tank versus growth day. As growth day

increased absorbance increased. Tank 3: Trt 2 and Tank 6: Trt 2 experienced a decrease in absorbance at day 32 compared to the other tanks.



Trt means treatment. Treatment 1 average scalar irradiance of 1041 ± 269.18 µmol m-2 s-1 PAR Treatment 2 ASI of 430 ± 96.03 µmol m-2 s-1 PAR. provided optical density for cocultures, displays of absorbance OD_{620} vs. growth day.

Figure 3.3. Optical Density of CCA

Table 3.2. Optical Density of CCA

Growth Day	0	7	13	22	25	30	32
Tank 1: Trt 1	0.075	0.100	0.077	0.150	ND	ND	ND
Tank 2: Trt 1	0.080	0.099	0.083	0.143	ND	ND	ND
Tank 3: Trt 1	0.067	0.101	0.165	0.363	0.487	0.664	0.278
Tank 4: Trt 2	0.060	0.071	0.080	0.125	0.203	0.333	0.347
Tank 5: Trt 2	0.070	0.079	0.157	0.224	0.263	0.309	0.350
Tank 6: Trt 2	0.068	0.092	0.180	0.247	0.294	0.285	0.241

Trt means treatment. Treatment 1 average scalar irradiance of 1041 ± 269.18 µmol m-2 s-1 PAR Treatment 2 ASI of 430 ± 96.03 µmol m-2 s-1 PAR. expressed in absorbance at 620nm

3.3.4. pH

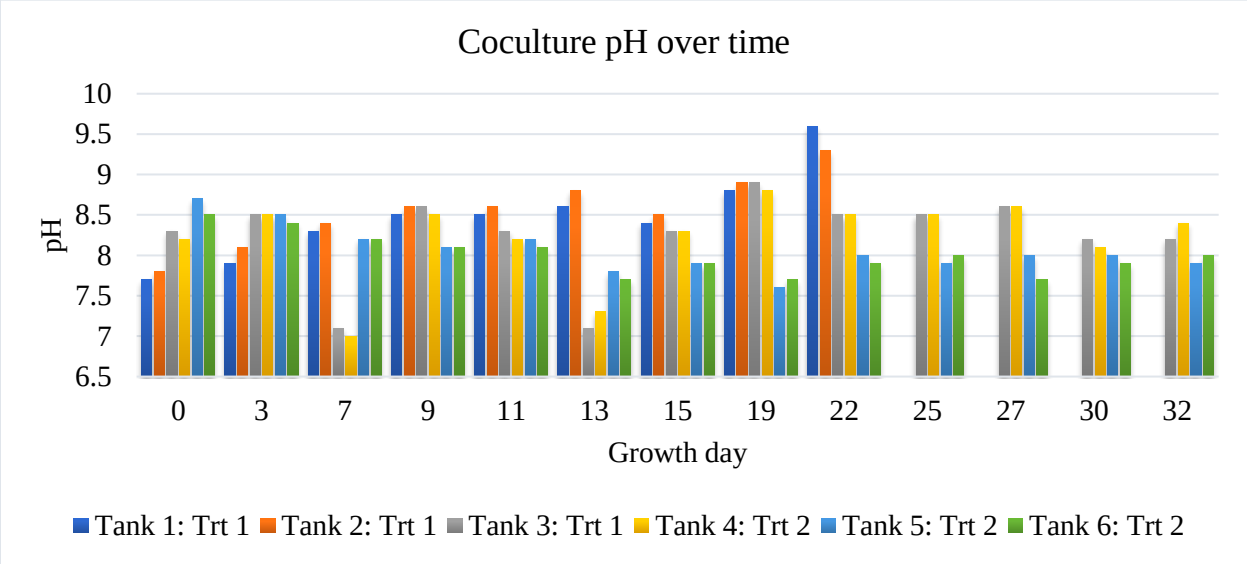
The cocultures pH fluctuated from 7.0-8.1 during the initial log and exponential growth phases but as the cells matured the pH increased and required dosing with CO_2 as seen in Table 3.3. In previous studies of *Chlorella* sp., it was found that biomass and lipid productivities were highest at pH 7.5 by Moheimani (2013). After exponential growth phase cultures maintained a

higher pH (7.5-8.5) even after dosing with CO_2 to lower the pH to 6-6.5 cultures stabilized at about pH 8.0 within 2 days, this is due to the cultures reaching stationary phase. Trt 1 pH was slightly increased compared to Trt 2 this can be attributed to the culture growing rapidly releasing oxygen and using CO_2.

Table 3.3. Average CCA pH

	Average	Std. Dev.
Tank 1: Trt 1	8.5	0.5
Tank 2: Trt 1	8.6	0.4
Tank 3 Trt 1	8.2	0.5
Tank 4: Trt 2	8.2	0.5
Tank 5: Trt 2	8.1	0.3
Tank 6: Trt 2	8.0	0.3

Trt means treatment. Treatment 1 average scalar irradiance of 1041 ± 269.18 µmol m-2 s-1 PAR and treatment 2 ASI of 430 ± 96.03 µmol m-2 s-1 PAR.



Treatment 1 average scalar irradiance of 1041 ± 269.18 µmol m-2 s-1 PAR (more light) and treatment 2 ASI of 430 ± 96.03 µmol m-2 s-1 PAR (less light).

Figure 3.4. CCA pH vs. Time

3.3.5. Temperature

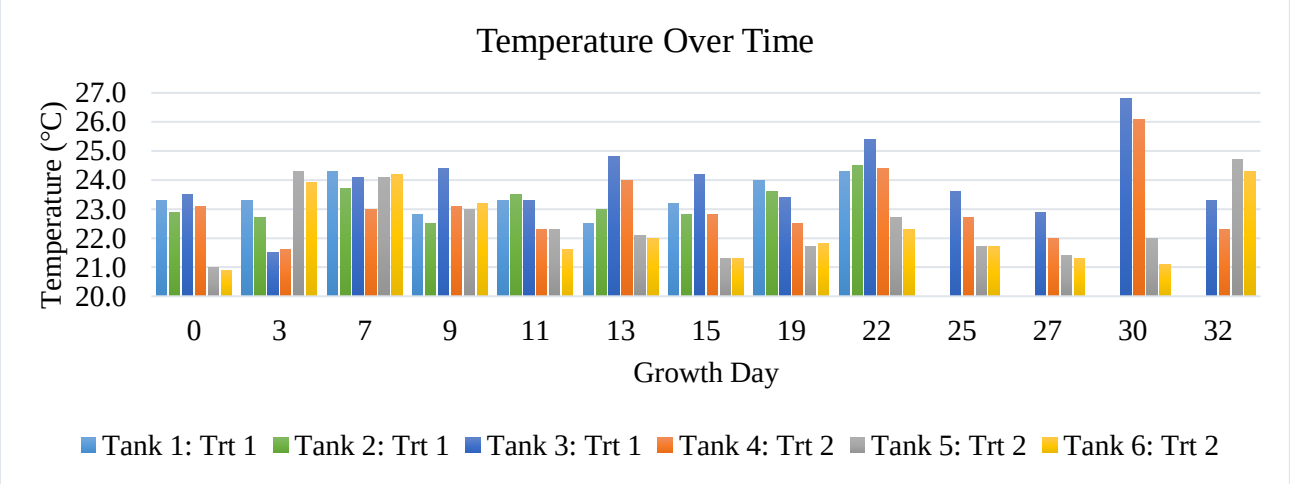
Coculture temperature was monitored as an environmental parameter over time in days (Figure 3.5). Tank 3, Tank 1 and Tank 2 which all received treatment 1 experienced the greatest

average temperatures at 23.9, 23.4, and 23.2°C, respectively. This is due to the lamp being 10" from the culture's surface and putting off heat that increased the cultures average temperature. There was no significant difference in coculture temperatures between treatments 1 and 2 at $p < .05$ ($p= 0.51$).

Table 3.4. Average CCA Temperature

	Average Temperature (°C)	Std. Dev.
Tank 1: Trt 1	23.4	0.6
Tank 2: Trt 1	23.2	0.6
Tank 3: Trt 1	23.9	1.3
Tank 4: Trt 2	23.1	1.2
Tank 5: Trt 2	22.5	1.2
Tank 6: Trt 2	22.3	1.2

Trt means treatment. Treatment 1 average scalar irradiance of 1041 ± 269.18 µmol m-2 s-1 PAR and treatment 2 ASI of 430 ± 96.03 µmol m-2 s-1 PAR.



Trt means treatment. Treatment 1 average scalar irradiance of 1041 ± 269.18 µmol m-2 s-1 PAR (more light) and treatment 2 ASI of 430 ± 96.03 µmol m-2 s-1 PAR (less light).

Figure 3.5. CCA Temperature vs. Time

3.4. Conclusion

Flow cytometry provided data on the cell concentration, and species ratio of CCA. *Chlorella vulgaris* L. was the dominant species in CCA, when compared to cyanobacteria

Leptolyngbya. This was the expected response from the irradiance levels each treatment received. CCA grew as expected in the conditioned parameters of pH, temperature, culture nutrients, aeration, and irradiance. The irradiance treatments applied to influence the *Chlorella*: cyanobacteria ratio was successful as the higher irradiance 1041 ± 269.18 μmol m-2 s-1 PAR (Treatment 1) produced an average culture ratio of 97.47 ± 1.29% *Chlorella vulgaris* L., and 2.84 ± 1.27% cyanobacteria *Leptolyngbya*. The lower irradiance 430 ± 96.03 μmol m-2 s-1 PAR (Treatment 2) produced an average culture ratio of 89.85 ± 1.17 *Chlorella vulgaris* L, and 10.64 ± 1.97 cyanobacteria *Leptolyngbya*. Trt 1 contained significantly more *Chlorella vulgaris* L at 95% confidence than trt 2 (p=0.001). Cyanobacteria *Leptolyngbya* in trt 2 was significantly higher at 95% confidence when compared to trt 1. It can be concluded from this study that two irradiance treatments successfully produced biomass with two different *Chlorella vulgaris* L and cyanobacteria *Leptolyngbya* culture ratios for the biomass phase of this study.

Future studies could cultivate isolated, axenic CCA in closed reactors under specific light regimes, and photoperiods to better assess growth parameters effect on bioactive compounds of interest like the blue and red photo exposure in Barnett and others (2015) and the irradiance experiments by Bai (2012) and Silaban (2013).

3.5. References

Bai, R. (2012). Lipid Production from a Louisiana Native *Chlorella vulgaris*/*Leptolyngbya* sp. Co-culture for Biofuel Applications. Chemical Engineering Commons. LSU Doctoral Dissertation

Barnett, J. Z., Foy, J., Malone, R., Rusch, K. A., & Gutierrez-Wing, M. T. (2017). Impact of light quality on a native Louisiana *Chlorella vulgaris* L./*Leptolyngbya* sp. co-culture. Engineering in Life Sciences, 17(6), 678-685.

Benson, B.C., Rusch, K.A. (2006) Investigation of the light dynamics and their impact on algal growth rate in a hydraulically integrated serial turbidostat algal reactor (HISTAR). Aquacultural engineering 35, 122-134.

Bold, H.C., 1949. The Morphology of Chlamydomonas Chlamydogama, sp. Nov. Bulletin of the Torrey Botanical Club, 76(2), pp.101-108.

Brown, R.M., Jr., & Bold, H.C. (1964). Comparative studies of the algal genera Tetracystis and Chlorococcum. University of Texas Publication No. 6417: 1-213.

Gutierrez-Wing, M.T, (unpublished work). Notes from meetings. Verbal information provided based on experience. Department of Civil and Environmental Engineering. Louisiana State University.

Hyka, P., Lickova, S., Přibyl, P., Melzoch, K., & Kovar, K. (2013). Flow cytometry for the development of biotechnological processes with microalgae. Biotechnology advances, 31(1), 2-16.

Mendoza, H., De la Jara, A., Freijanes, K., Carmona, L., Ramos, A. A., de Sousa Duarte, V., ... & Carlos, J. (2008). Characterization of Dunaliella salina strains by flow cytometry: a new approach to select carotenoid hyperproducing strains. Electronic Journal of Biotechnology, 11(4), 5-6.

Moheimani, N.R. J Appl Phycol (2013) 25: 387. https://doi.org/10.1007/s10811-012-9873-6

Pulz, O. (2001). Photobioreactors: production systems for phototrophic microorganisms. Applied microbiology and biotechnology, 57(3), 287-293.

Silaban, A. G. (2012). Growth rate and productivity of a Louisiana native microalgae/cyanobacteria co-culture: Feasibility for use in industrial biotechnology applications. Louisiana State University Graduate School Digital Commons. Dept of Civil and Environmental Engineering. Master's Thesis.

Starr, R. C., & Zeikus, J. A. (1993). Utex—The Culture Collection of Algae at The University of Texas At Austin 1993 List of Cultures 1. Journal of Phycology, 29, 1-106.

Tate, J. J., Gutierrez-Wing, M. T., Rusch, K. A., & Benton, M. G. (2013). Gene expression analysis of a Louisiana native *Chlorella vulgaris* L. (Chlorophyta)/*Leptolyngbya* sp.

(Cyanobacteria) co-culture using suppression subtractive hybridization. Engineering in Life Sciences, 13(2), 185-193. doi:10.1002/elsc.201200063

UTEX Culture Collection of Algae. (2016). Bold 1NV Medium. Algal Media Recipes. https://utex.org/products/bold-1nv-medium.

Zhang, X. (2015). Microalgae removal of CO2 from flue gas. Clean coal technology research reports, April. Retrieved from http://bookshop. iea-coal. org. uk/reports/ccc-250/83697, 244.

Chapter 4. Protein Characterization of Louisiana Native Co-Culture of Microalgae (*Chlorella Vulgaris* L.) and Cyanobacteria (*Leptolyngbya* sp.)

4.1. Introduction

Microalgae is a source of functional ingredients with positive health effects due to high PUFAs, polysaccharides, pigments, minerals, vitamins, enzymes and bioactive peptides (Capelli and Cysewki, 2010). Algal proteins are chiefly enzymatic proteins (Becker, 2007). *Chlorella vulgaris L.* is reported to have 51-58% protein and *Arthospira* platensis (cyanobacteria sp.) is reported to have 46-63% protein DWB (Becker, 2007). There is research to suggest that *Chlorella* protein hydrolysate has shown immune enhancing activity in mice and can possibly be used for developing functional foods (Morris and others, 2007). *Chlorella vulgaris L.* proteins have emulsifying capabilities (Ursu and others, 2014). Cyanobacteria, red algae and cryptomonads contain phycobiliproteins that are fluorescent photosynthetic complexes (Glazer, 1989; Glazer in 1994).

Protein content varies based on culture type and growth conditions. Little is known about algal protein chemical properties. A better understanding of the proteins present in algae strains is needed if algal proteins are to be applied in food products and medical applications. There is a consumer trend for high-protein foods and CCA proteins could possibly be used as a source of "green" or vegan proteins and nutraceuticals. There is a growing demand for healthy, tasty, sustainable, low impact, high-protein foods. Microalgal products need to become more diversified and economically competitive. Algal proteins could possibly be used as a source of "green" or vegan proteins and nutraceuticals. Microalgae proteins are multifaceted, valuable and competitive in the consumer market. The objective of this study was to characterize proteins in

Chlorella vulgaris (Chlorophyta)/*Leptolyngbya* sp. (Cyanobacteria) co-culture microalgae (CCA).

4.2. Materials/Experimental Design

Louisiana native co-culture of microalgae (*Chlorella vulgaris* L.) and cyanobacteria (*Leptolyngbya* sp.) (CCA) was provided by Dr. Gutierrez-Wing in the Aquatic Germplasm and Genetic Resources center of the School of Renewable Natural Resources, LSU Ag Center. The CCA was cultivated using the growth parameters: scalar irradiance, pH 7-9, temperature 25 ± 2°C, Bold 1NV growth media, and 40 LPM (liters per minute) aeration. Two irradiance treatments were applied to CCA in this study. CCA was cultivated in 6 cultures, 3 cultures were exposed to average scalar irradiance (ASI) 1041 ± 269.18 μmol m^{-2} s^{-1} PAR and will be referred to as Treatment 1 and the other 3 cultures were exposed to ASI 430 ± 96.03 μmol m^{-2} s^{-1} PAR and will be referred to as a Treatment 2 .

Algae was harvested during stationary growth phase which is determined as on the first day of decrease in optical density, using a semi-continuous flow centrifuge at 2L/min. All cultures were frozen after harvest and stored at 4°C short-term (2 months) or at -20˚C long-term (3-12 months).

The experiment was conducted in a completely randomized design (CRD), with no blocks. There were 6 tanks chosen at random to contain one of the two treatment levels, Treatment 1 – cultures exposed to ASI of 1041 ± 269.18 μmol m^{-2} s^{-1} PAR and Treatment 2– cultures exposed to ASI 430 ± 96.03 μmol m^{-2} s^{-1} PAR. The co-culture species ratio (*Chlorella*: Cyanobacteria) was the response of the treatments. The experimental and sampling units were the algae tanks.

Statistical analysis was performed using two-sample t-test (GraphPad, QuickCalc) available at https://www.graphpad.com/quickcalcs/ . The two-sample t-test was performed at 95% confidence to determine the difference in the two treatments, this test compared the average means of each dependent variable (assay performed).

Lowry and Dumas method provided total protein content, HPLC PDA provided amino acid profile, SDS-PAGE provided molecular mass of extracted proteins. MALDI-TOF-MS identified several peptides in CCA.

4.3. Protein Characterization Methods

4.3.1. Lyophilization of Algae Samples

Algae samples were prepared by lyophilization for subsequent assays. This process is based on sublimation of water occurs that occurs at pressures and temperature below the triple point of 4.6 mm of Hg and 0.0099 °C (Bhambere and others, 2015). Harvested algae paste (cells centrifuged to remove culture water) was weighed into plastic weigh boats. Aluminum foils covered weigh boats and samples. Samples were frozen at -20°C for 12 h, then further frozen at -80°C for 12 h so that when frozen semi-liquid sublimates would leave only solid, dried components of the original semi-liquid. The aluminum foil on top of the samples was carefully perforated with a needle or pin. Samples were placed in the Genesis 35XL pilot lyophilizer (Stone Ridge, NY), under a vacuum, sublimating the ice directly into water vapor. The freeze dryer condensed the water vapor for at least 24-48 h depending on the sample weights, and moisture content of the initial sample. After the ice in the algae sample was completely sublimated, samples were removed from the machine and machine care and standby protocol was initiated (Bhambere and others, 2015). Samples were transferred into freezer bags and stored in a desiccator at -20°C until needed for assays.

4.3.2. Total Protein Content

Lyophilized, prepared algae samples were analyzed for protein content using the Lowry assay, and Dumas method. Lowry method has been previously researched and found to be the optimal method to quantify algal protein content (Gutierrez and others, unpublished work).

4.3.2.1 Sample Preparation

Slocombe and others (2013) analyzed seven species of microalgae for rapid protein measurement, they found that hot TCA extraction conditions (24% (w/v) TCA at 95 °C) enhanced yields for three out of seven strains of algae compared with milder treatments. That study proposed that this treatment is widely applicable in microalgae. Based on Slocombe and others (2013) study a 5mg sample of lyophilized algae was added to 0.2 mL 24% (w/v) TCA, incubated at 95°C for 15 min to cause TCA precipitation. Samples were cooled to room temperature then 0.6 mL of DI water was added; samples were then centrifuged for 20 min at 4°C. The supernatant was discarded, and the pellet was added to 0.5 mL Lowry reagent D and vortexed to create an alkaline suspension, this solution was incubated at 55°C for 3h then centrifuged for 20 min at room temp. The pellet was discarded, and the supernatant was retained for the protein assay (Price, 1965; Slocombe and others, 2013).

4.3.2.2. Total Protein Content by Lowry Assay

The Lowry solution was prepared fresh, and consisted of Sol A, Sol B and, Sol C in the following ratio 100:1:1. Solution A was an alkaline solution containing 2.86g NaOH and 14.31g Na_2CO_3 in 500mL with DI water. Solution B contained 1.42g of $CuSO_4 \cdot 5(H_2O)$ this solution was in 100mL with DI water. Solution C contained 2.85g of Na_2Tartrate$\cdot 2(H_2O)$ in 100mL with DI water. This Lowry's solution was light sensitive, so it was made in the last 5 minutes of sample incubation and stored in an amber bottle (or foil wrapped bottle). Protein concentrations were

quantified with reference to standards of bovine serum albumin (BSA). BSA standards were a range of 1 to 100 µg protein to a volume of 1 ml. The samples were added to water in a 16 × 125–mm test tube to yield a final volume of 1 ml. Two separate tubes containing water were included for water blanks. Buffer blanks were also used. Five milliliters of the freshly prepared Lowry solution were added to each tube and thoroughly vortexed. Tubes were then incubated for 10 min at room temperature, then 0.5 ml of diluted Folin-Ciocalteu reagent was added to each tube and vortexed immediately. Tubes were incubated an additional 30 min at room temperature and the absorbance was read at 750nm. A standard curve was created using data from the standard protein. The concentration of unknown algae sample protein was calculated from the standard curve equation (Lowry and others, 1951; Neilson, 2010).

4.3.2.3. Total Protein Content by Dumas Method

A algae sample of about 1 g was placed in a ceramic crucible. The crucible was placed in a furnace (1050 ◦C) with an atmosphere of oxygen. The algae sample was burned, and the organic elements were oxidized. The combustion gases were collected and passed through several traps. All gases were disregarded except nitrogen and nitrogen oxides. An aliquot (10 ml) of that gas was carried by helium gas over a copper catalyst to convert the nitrogen oxides. The mixture was then carried into a thermal-conductivity cell that produced an electrical signal relative to the nitrogen content. The result was calculated from a calibration curve plotted using known glycine standards and expressed as a percentage of the initial sample weight (Saint-Denis and others, 2004). The conversion factor of various species of algae was previously determined by López and others (2010) and further validated by Lourenço and others (2012); for this project, a conversion factor of 5.35 was used (Neilson, 2010).

4.3.3. Protein Profile by SDS-PAGE

4.3.3.1. Sample Preparation

Lyophilized CCA was dispersed in DI water to create a suspension (10% w/w dry mass). The algae suspension was sonicated on ice for 15 min pulsing 15 secs resting 10 secs to limit protein damage during extraction. The CCA suspensions were centrifuged at 4°C, 5000 RPM for 30 min. The supernatants containing proteins were used for further SDS-PAGE analysis (Ursu and others, 2014).

4.3.3.2. SDS-PAGE Methodology

Electrophoresis was used to separate and visualize proteins. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), proteins were separated based on size. Protein extracts were applied to gels. The visualization of the proteins made it possible to distinguish between different types of algae since most algal species have a distinguishing protein pattern (Nielson, 2010).

In this experiment, proteins were extracted with 2.5% SDS, in serial dilutions (1, 10, 100 and 1000) proteins bonded to SDS becoming highly negatively charged and moved through the gel matrix toward the anode at a rate based on size. The molecular mass of protein subunits was estimated by comparing its mobility with protein standards. The molecular mass of extracted proteins was determined under denaturing conditions SDS–PAGE according to Schägger and von Jagow, (1987). For one-dimensional electrophoresis Novex™ Sharp pre-stained standards (Thermo Fisher) were used with a range of 3.5-200 kDa (Nielsen, 2010).

4.3.4. Amino Acid Sequencing by MALDI-TOF MS

MALDI-TOF-MS analysis was used to determine the amino acid sequence of peptide fragments present in solubilized CCA algae proteins. Protein extracts were prepared from SDS-

PAGE gel bands of interest. MALDI consisted of nanoscale liquid chromatography coupled with a collector that deposited micro-fractions on a MALDI plate, a mass spectrometer analyzed the fractions. For the MS analysis, a sample solution was prepared by adding 10 μL 0.1% trifluoroacetic acid (TFA) in water /acetonitrile (50/50, v/v) to each of the protein gel band samples. A saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in a mixture of 50/50 (v/v) acetonitrile and 0.1% TFA in water, and this solution was used as the matrix. A sample solution of 0.5 μL was deposited onto the MALDI plate followed by 0.5 μL matrix deposition above it and this was mixed before the drying of the components. The thin layer of matrix absorbs energy and then the sample is analyzed to produce an ordered array of mass spectra, each containing m/z values (Caprioli and others, 1997). MALDI-TOF MS measurements were performed on a commercial instrument (Ultraflextreme, Bruker Daltonics, Billerica, MA, USA). Mass spectra was recorded in positive ion reflectron mode with an accelerating voltage of 25 kV and analyzed in the mass range of 500–4500 Da. The spectra were acquired after calibration of the instrument with a peptide standard (Peptide Calibration Standard II, Bruker Daltonics, MA, USA). A minimum of 500 laser shots per sample was used to generate each mass spectrum (Barbano and others, 2015).

4.3.5. Amino Acid Determination by HPLC PDA

Amino acids were identified by HPLC PDA. Tryptophan, Methionine, and Cysteine degrade at the extraction temperature. Ammonia content was considered for hydrolyzed products of glutamate and asparagine (glutamic acid, and aspartic acid) (Mossé 1990; Yeoh and Truong 1996).

4.3.5.1. Sample Preparation

About 25 mg of lyophilized algae sample was weighed into a hydrolysis tube and 0.8 ml of 6N HCl containing 0.25% phenol was added. The sample was frozen under vacuum for 2 min then thawed, this was repeated 3 times. This step occurred in the hydrolysis tube, the tube was connected to a vacuum tube/spicket to remove oxygen from the tube headspace and sample. The tube was placed in a small amount of liquid nitrogen for 2 min, the tube and sample were then allowed to thaw; this lysed the algae cell wall. The tube was placed on a heating block to further hydrolyze for 24 h at 110 °C. The hydrolysate was transferred and washed into a 5 mL volumetric flask and brought up to volume. Then 200 µl of this solution was mixed with 20 ul of 2.5 umol/ml norleucine (internal standard) then dried. Then 100 ul PITC solution (EtOH: water: PITC: triethylamine = 7:1:1:1) was added to the residue and mixed for 30 min. The sample was then freeze dried using the method outlined previously in sample preparation using a Genesis 35XL pilot lyophilizer (Stone Ridge, NY) (Lourenço and others 2002). The derivatized residue was dissolved in 2 ml of buffer (140 mM sodium acetate, 0.05% triethylamine, titrated to pH 6.40 with glacial acetic acid with the addition of 60 ml/L acetonitrile) and filtered with 0.2 um filter to obtain the injection sample (Barbarino, and Lourenço, 2005). Samples are not commonly purified for amino acid hydrolysis, they are derivatized.

4.3.5.2. System Conditions

The amino acid analysis was performed with a Dionex Ultimate-3000 system, which included a Dionex Ultimate 3000 Pump, Ultimate 3000 Autosampler, Ultimate 3000 Column Compartment, and Ultimate 3000 Photodiode Detector. Chromeleon 6.8 software was used to control the system and process data. The sample was separated on a Waters Pico-Tag C18 column (4um, 3.9 x 150 mm) with Nova-Pak guard column (4 µm, 3.9 x 20 mm) maintained at

38 °C. The mobile phase consisted of eluent A (140 mM sodium acetate, 0.05% triethylamine, titrated to pH 6.40 with glacial acetic acid with the addition of 60 ml/L acetonitrile) and eluent B (60% acetonitrile in water). The detected wavelength was set at 254 nm. Injection volume was 20 ul. Amino Acid determination followed the steps outlined by Dhillon and others in 2014.

4.3.5.3. Standard Curve

Standard amino acids were purchased from Thermo Scientific, with 2.5 μmol/ml for each amino acid in 0.1 N HCl (alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, and valine) and 1.25 μmol /ml for cystine. Norleucine was the internal standard and purchased from Sigma. The stock solution of amino acid standards was prepared as 200 μmol/ml (cystine was 100 nmol/ml) because cystine is an oxidized dimer of 2 cysteine molecules that are connected through a disulfide bond. The amino acid standard was diluted as a series of solutions at 100, 50, and 25 nmol/ml. They were run to make a calibration curve (Barbarino, and Lourenço, 2005).

4.4. Results and Discussion

4.4.1. Total Protein Content

The Lowry Assay found that Treatment 1 contained 29.46 ± 6.11 g protein per 100 g of algae and Treatment 2 contained 39.67 ± 5.15 g protein per 100 g of algae (Table 4.1.). Treatment 2 contained significantly more protein at 95% confidence ($p = 0.001$). This result was expected since Treatment 2 CCA contained more cyanobacteria that is protein rich (Kim and others, 2015) compared to *Chlorella*.

The Dumas Assay found that Treatment 1 was 34.63 ± 1.54 g protein/ 100 g algae DWB by and treatment 2 was 34.65 ± 6.63 g protein/ 100 g algae DWB. There was no significant

difference in percent protein treatments at 95% confidence (p = 0.99). The Dumas method measures all nitrogen present in the algae samples and therefore isn't a true measure of protein. Lowry method measures hydro-soluble, intracellular and extracellular proteins when the algae sample is pretreated to lyse the cell wall (Ebeling, 1968). Due to its accuracy and ease of use the Lowry method and modifications of it are the most commonly used protein content methods in algae (Peterson, 1979; Barbarino and Laurenco, 2005; Cerón and others, 2008; López and others, 2010; Ursu and others, 2014; Safi and others, 2014). The high protein content of Lowry method for Trt 2 39.67± 5.15 g protein/ 100 g algae is likely due to some residual algal pigments (chlorophyll a/b, c- phycocyanin) interfering with the absorbance reading.

Table 4.1 Total Protein Content of CCA

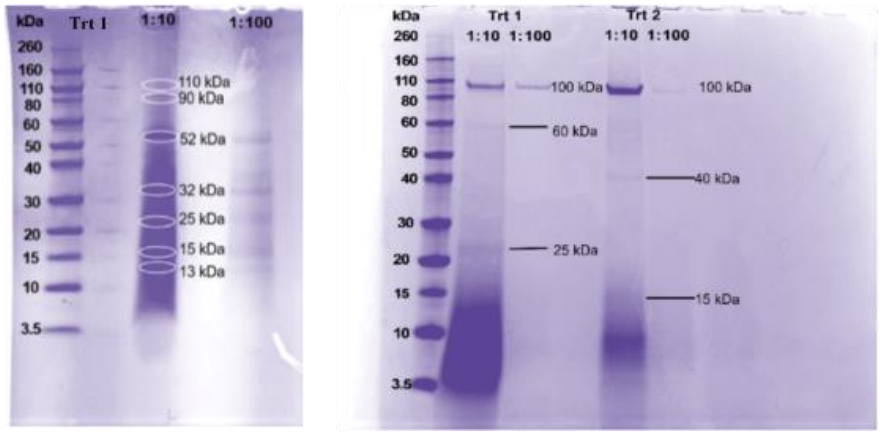
Treatment	Assay	Total Protein Content (g protein/ 100 g algae)
1	Lowry	29.46 ± 6.11
2	Lowry	39.67± 5.15
1	Dumas	34.63 ± 1.54
2	Dumas	34.65 ± 6.63

Treatment 1 average scalar irradiance of 1041 ± 269.18 μmol m-2 s-1 PAR. Treatment 2 ASI of 430 ± 96.03 μmol m-2 s-1 PAR.

4.4.2. Protein Profile by SDS-PAGE

Protein profile in algal samples were qualitatively analyzed by SDS-PAGE (Laemmli, 1970; Ursu and others, 2014, Neilson, 2010). Bands were identified at 100-110, 90, 60-52, 33-32, 40, 25, 15, and 13 kDa. These bands can be seen in Figure 4.1. The 52, and 15 kDa were hypothesized as L8S8 RUBISCO enzyme (Roy and Cannon, 1988). RUBISCO has a molecular mass of ~560 kDa and consists of 8 small (~14 kDa each) and 8 large (~56 kDa each) subunits arranged as 8 heterodimers (Malkin and Niyogi, 2000). Rubisco was also found in the coculture previously by Silaban and others (2012). A study on *Chlorella vulgaris* by Kairy and others (2011) used SDS-PAGE to find the M_w of two unidentified proteins at 75 and 39 kDa. Swanson

and Glazer (1990) identified several phycobiliprotein subunits that ranged from 7.5 to 30 kDa, so the 13 kDa band identified in CCA could potentially contain these phycobiliprotein subunits.



Treatment 1 average scalar irradiance of 1041 ± 269.18 µmol m-2 s-1 PAR. Treatment 2 ASI of 430 ± 96.03 µmol m-2 s-1 PAR.

Figure 4.1. SDS-PAGE of Extracted CCA Peptides

4.4.3. Amino Acid Sequencing by MALDI-TOF MS

MALDI-TOF-MS provided a mass to charge (m/z) value and peptide sequence that when paired with the MASCOT and UNIPROT databases identified several enzymes, ATP binding subunits, heat shock proteins, centriole spindle associated partial proteins (related to bacteria in CCA), transcriptional regulators and, uncharacterized proteins. A comprehensive list of proteins from the databases were compiled by matching percentage and, frequency. This list was summarized in Figure 4.2., that focuses on the most frequent specific protein types found in each SDS-PAGE band. Appendix A displays all SDS PAGE band MALDI-TOF-MS peptide sequences, spectra and MASCOT and UNIPROT findings. These findings confirm Tate and others (2013) research that identified molecular chaperone/heat shock protein family, and ATP synthase subunits in primers using qPCR in CCA.

In the 13 kDa band DoxD family protein/pyridine nucleotide-disulfide oxidoreductase was found, it is a part of protein coding and oxidative phosphorylation. The pyridine nucleotide-

disulphide oxidoreductases are FAD (flavin adenine dinucleotide) flavoproteins which contain two redox-active cysteines (Kuriyan and others, 1991). In the 15 kDa band LysR transcriptional regulators were identified, they control genes involved in virulence, metabolism, quorum sensing and motility (Maddocks and Oyston, 2008).

In the 32 kDa band Tryptophan synthase alpha chain (EC 4.2.1.20) was identified. This synthase is required for tryptophan biosynthesis and verifies that tryptophan is present in CCA even though the amino acid determination didn't quantify Tryptophan due to the temperature used during extraction and hydrolysis. His Kinase A (Phospho-acceptor) domain-containing protein is a membrane bound signal transducer, and is used to sense environmental stimuli, they regulate cellular response in bacteria (Stock and others, 2000). 2Fe-2S ferredoxin is found in chloroplast membranes and functions as an electron carrier in the photosynthesis electron transport chain, 2Fe-2S ferredoxin also donates electrons to other cellular proteins (Rypniewski and others, 1991). This indicates the CCA proteins are active and regulating cellular responses during cultivation. These peptides are components of the photosynthesis process, identifying them in CCA may aid in understanding biosynthesis of valuable compounds.

Raven and Beardall (2003) hypothesized that algae contain alternative oxidase, and cytochrome oxidases. Tang and Satoh (1985) identified oxidoreductase in cyanobacteria, they found that core complexes have a corresponding oxygen producing polypeptide at 33 kDa. In this study oxidoreductase was identified in the 13 kDa and 25 kDa SDS-PAGE band of CCA. Two other membrane related proteins found in higher plants and *Chlorella* are 23 kDa, and 16 kDa and they have a role in the oxygen optimizing capacity of the organisms they are found in (Kuwabara and Murata, 1979Åkerlund, 1982; Bricker and others, 1988; Bricker and Frankel, 1998; 2002; Burnap and others, 1992; Wydrzynski and Satoh, 2005).

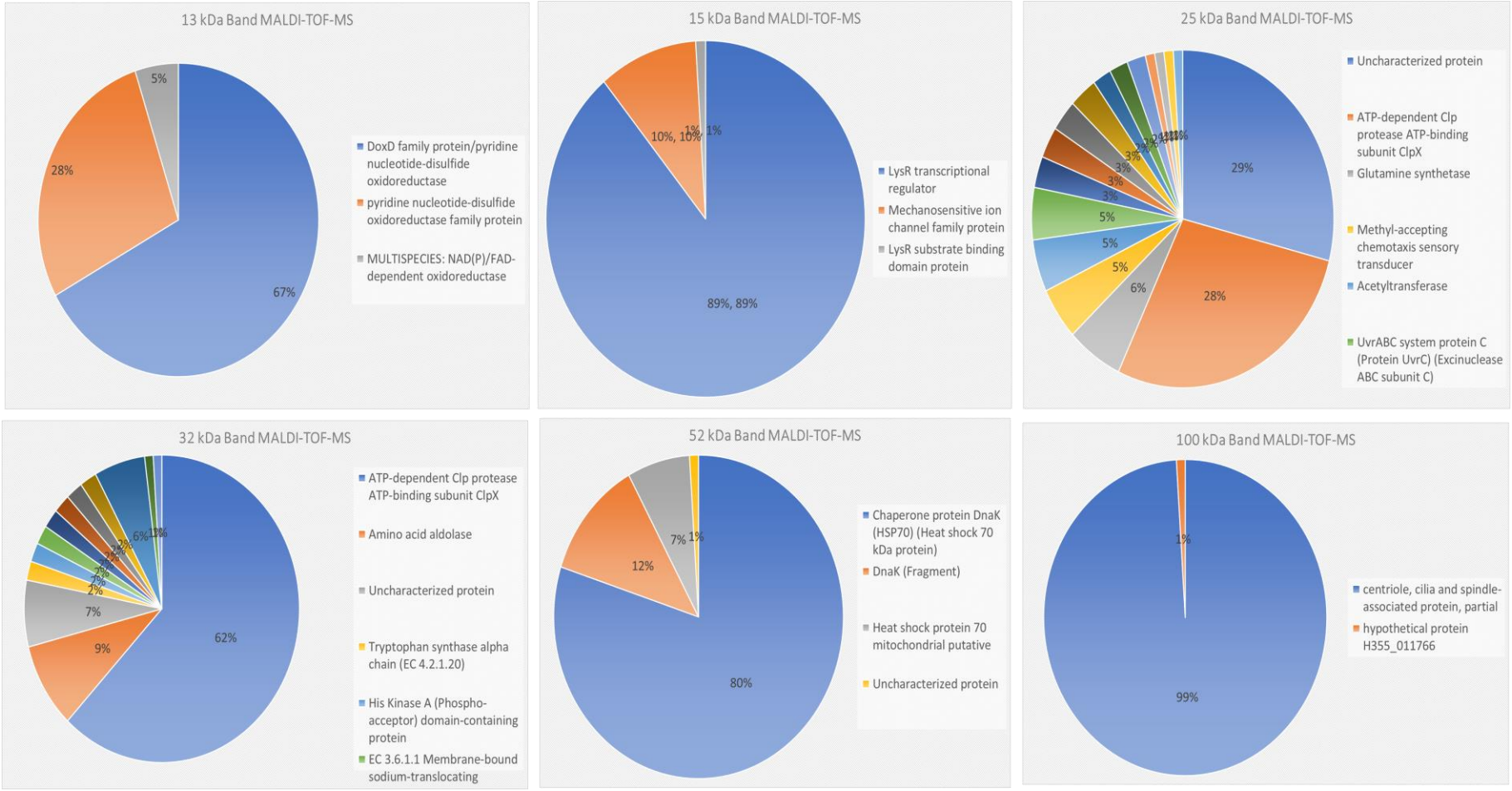


Figure 4.2. Proteins Identified in CCA SDS-PAGE Bands

4.4.4. Amino Acid Determination by HPLC PDA

Seventeen out of 21 amino acids (AA) were detected in each treatment of CCA. CCA contained all the essential AA making it a complete protein. Treatment 1 contained 29.41 ± 1.20 g total amino acids per 100 g of algae DWB, and 30.28 ± 2.80 g total AA per 100 g of algae DWB for treatment 2 seen in Table 4.2. The most prevalent AA were Alanine (Ala), Glutamine as Glutamic Acid (Glx) and Asparagine as Aspartic Acid (Asx) respectively in Trt 1 and Glx, Asx, and Ala respectively in Trt 2. There was no significant difference in amino acid content between treatments at 95% confidence ($p = 0.66$). Tryptophan was not measured because it degrades at the extraction temperature used, but tryptophan has been identified in *Chlorella* and Cyanobacteria sp. separately based on previous studies (Ursu and others, 2014; Kim and others, 2015).

Mohtashamian (2012) found very similar AA profile in CCA grown at ASI 400 μmol s^{-1} m^{-2}, they compared culture dilution rates of 0.360 0.459 0.558 d^{-1}, and pre-/post- lipid extraction effect on amino acid content of CCA. Mohtashamian found that CCA contained 27.4 - 46.1 g/ 100g of amino acids. The amino acid content found was comparable to that of Kim and others (2015) where they found Cyanobacteria *Leptolyngbya* sp. contained 33.44 g AA per 100 g of algae, the most prevalent being Glutamic acid, Aspartic acid, and Alanine. In order to obtain the quantity of AAs in total protein content (TPC) research on the association between the contents was necessary. A study on the complete amino acid profile of beef found that the AA profile amounted to 91% of protein based on total nitrogen (Hall and Schönfeldt, 2013). This data follows that tendency as well when comparing total AA content to Lowry total protein content of CCA. Through HPLC-PDA amino acid content provides 80-90% of the TPC of CCA. In this study it can be estimated amino acids that weren't quantified or were under quantified are

attributed to extraction time/ high temperature (Tryptophan, Methionine, and Cysteine) account for the discrepancy in % of TPC.

Table 4.2. Average Amino Acid Content in CCA

	Treatment 1				Treatment 2			
AA	Average Content (g AA /100 g algae DWB)			Average Content in CCA (%)	Average Content (g AA /100 g algae DWB)			Average Content in CCA (%)
Ala	3.23	±	0.12 a	0.3 ± 0.0	2.98	±	0.28 a	0.3 ± 0.0
Glx	3.12	±	0.18 a	0.3 ± 0.0	3.40	±	0.56 a	0.3 ± 0.1
Asx	2.70	±	0.35 a	0.3 ± 0.0	3.17	±	0.49 a	0.3 ± 0.0
Leu*	2.66	±	0.17 a	0.3 ± 0.0	2.80	±	0.28 a	0.3 ± 0.0
Gly	2.34	±	0.21 a	0.2 ± 0.0	2.46	±	0.16 a	0.2 ± 0.0
Arg	2.30	±	0.54 a	0.2 ± 0.1	1.85	±	0.21 a	0.2 ± 0.0
Pro	2.09	±	0.28 a	0.2 ± 0.0	2.41	±	0.04 a	0.2 ±0.0
Lys*	1.79	±	0.08 a	0.2 ± 0.0	1.65	±	0.17 a	0.2 ±0.0
Thr*	1.72	±	0.12 a	0.2 ± 0.0	1.84	±	0.10 a	0.2 ±0.0
Ser	1.68	±	0.22 a	0.2 ± 0.0	1.93	±	0.03 a	0.2 ±0.0
Val*	1.65	±	0.11 a	0.2 ± 0.0	1.60	±	0.19 a	0.2 ±0.0
Phe*	1.51	±	0.08 a	0.2 ± 0.0	1.53	±	0.13 a	0.2 ±0.0
Ile*	0.95	±	0.08 a	0.1 ± 0.0	0.93	±	0.14 a	0.1 ±0.0
Tyr	0.89	±	0.05 a	0.1 ± 0.0	0.98	±	0.05 a	0.1 ±0.0
Met*	0.49	±	0.06 a	0.0 ± 0.0	0.44	±	0.04 a	0.0 ±0.0
Cys	0.17	±	0.03 a	0.0 ± 0.0	0.21	±	0.04 a	0.0 ±0.0
His*	0.12	±	0.01 a	0.0 ± 0.0	0.10	±	0.02 a	0.0 ±0.0
Total	29.41	±	1.20 a	2.9 ± .0.1	30.28	±	2.79 a	3.0 ±0.3

*****denotes essential amino acid. Treatment 1 average scalar irradiance of 1041 ± 269.18 μmol m-2 s-1 PAR. Treatment 2 ASI of 430 ± 96.03 μmol m-2 s-1 PAR. Like letters represent no significant difference at 95% confidence.

4.5. Conclusion

The Lowry Assay found that Treatment 1 contained 29.46 ± 6.11 g protein per 100 g of algae DWB, and Treatment 2 contained 39.67 ± 5.15 g protein per 100 g of algae DWB; Treatment 2 contained significantly more protein at 95% confidence ($p = 0.010$). Seventeen out of 21 AA (amino acids) were detected in both treatments of CCA. The molecular mass of

extracted proteins was determined under denaturing conditions by SDS–PAGE; bands were identified at 100-110, 90, 52, 33-32, 25, 15, and 13 kDa. The 52 and 15 kDa peptides are proposed subunits of the L8S8 Rubisco enzyme and phycobiliprotein. MALDI-TOF-MS identified several enzymes, ATP binding subunits, heat shock proteins, centriole spindle associated partial proteins, transcriptional regulators and, uncharacterized proteins from the SDS-PAGE gel bands. CCA had a complete protein, containing all the essential AAs. The biomass from treatment 1 contained 29.41 ± 1.20 g total amino acids per 100 g of algae DWB, and 30.28 ± 2.80 g total amino acids per 100 g of algae DWB for treatment 2. There was no significant difference in amino acid content between treatments at 95% confidence ($p= 0.66$). This indicates that the treatments do not significantly change amino acid abundance in CCA.

Algal chemical composition and bioactivity levels are species and sample specific. These co-culture algal proteins could be used as a source of "green" or vegan proteins and nutraceuticals. CCA may also be used as a supplemented dietary protein in fishmeal. Protein content recovered varies greatly depending on cell wall breakdown, sample preparation, and extraction method. Washing the cells after harvesting is suggested to avoid culture nitrogen from causing protein overestimation. Future studies could focus on identifying texture related proteins in CCA and exploring rheological properties in them since algal proteins can be emulsifiers, and texture aids.

4.6. References

Åkerlund, H. E., Jansson, C., & Andersson, B. (1982). Reconstitution of photosynthetic water splitting in inside-out thylakoid vesicles and identification of a participating polypeptide.

Barbarino, E., & Lourenço, S. O. (2005). An evaluation of methods for extraction and quantification of protein from marine macro-and microalgae. Journal of Applied Phycology, 17(5), 447-460.

Bhambere, D., A. Gaidhani, K., Harwalkar, M., & S. Nirgude, P. (2015). Lyophilization / Freeze Drying – A Review (Vol. 4).

Bricker, T. M., & Frankel, L. K. (2002). The structure and function of CP47 and CP43 in photosystem II. Photosynthesis research, 72(2), 131.

Bricker, T. M., Morvant, J., Masri, N., Sutton, H. M., & Frankel, L. K. (1998). Isolation of a highly active photosystem II preparation from Synechocystis 6803 using a histidine-tagged mutant of CP 47. Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1409(1), 50-57.

Bricker, T. M., Odom, W. R., & Queirolo, C. B. (1988). Close association of the 33 kDa extrinsic protein with the apoprotein of CPa1 in photosystem II. FEBS letters, 231(1), 111-117.

Burnap, R. L., Shen, J. R., Jursinic, P. A., Inoue, Y., & Sherman, L. A. (1992). Oxygen yield and thermoluminescence characteristics of a cyanobacterium lacking the manganese-stabilizing protein of photosystem II. Biochemistry, 31(32), 7404-7410.

Capelli, B., & Cysewski, G. R. (2010). Potential health benefits of spirulina microalgae. Nutrafoods, 9(2), 19-26.

Caprioli, R. M., Farmer, T. B., & Gile, J. (1997). Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS. Analytical chemistry, 69(23), 4751-4760.

Cerón, M.C., Campos, I., Sánchez, J.F., Acién, F.G., Molina, E., Fernández-Sevilla, J.M., 2008. Recovery of lutein from microalgae biomass: development of a process for Scenedesmus almeriensis biomass. J. Agric. Food Chem. 56, 11761–11766.

Dhillon, M. K., Kumar, S., & Gujar, G. T. (2014). A common HPLC-PDA method for amino acid analysis in insects and plants. http://nopr.niscair.res.in/handle/123456789/25161

Ebeling, M. E. (1968). The Dumas method for nitrogen in feeds. Journal of the Association of Official Analytical Chemists, 51, 766-770.

Fowden L. (1952). The composition of the bulk proteins of *Chlorella*. The Biochemical journal, 50(3), 355–358.

Gutierrez-Wing, M.T, (unpublished work). Notes from meetings. Verbal information provided based on experience. Department of Civil and Environmental Engineering. Louisiana State University.

Hall, N. G., & Schönfeldt, H. C. (2013). Total nitrogen vs. amino-acid profile as indicator of protein content of beef. Food Chemistry, 140(3), 608-612. doi: https://doi.org/10.1016/j.foodchem.2012.08.046

Khairy, H. M., Ali, E. M., & Dowidar, S. M. (2011). Comparative effects of autotrophic and heterotrophic growth on some vitamins, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, amino acids and protein profile of *Chlorella vulgaris* Beijerinck. African Journal of Biotechnology, 10(62), 13514-13519.

Kim, J. H., Choi, W., Jeon, S.-M., Kim, T., Park, A., Kim, J., . . . Kang, D.-H. (2015). Isolation and characterization of *Leptolyngbya* sp. KIOST-1, a basophilic and euryhaline filamentous cyanobacterium from an open paddle-wheel raceway Arthrospira culture pond in Korea. Journal of Applied Microbiology, 119(6), 1597-1612. doi:10.1111/jam.12961

Kuriyan, J., Krishna, T. S. R., Wong, L., Guenther, B., Pahler, A., Williams, C. H., & Model, P. (1991). Convergent evolution of similar function in two structurally divergent enzymes. Nature, 352(6331), 172-174. doi:10.1038/352172a0

Kuwabara, T., & Murata, N. (1979). Purification and characterization of 33 kilodalton protein of spinach chloroplasts. Biochimica et Biophysica Acta (BBA)-Protein Structure, 581(2), 228-236.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, London 227, 680-685.

López, C. V. G., García, M. D. C. C., Fernández, F. G. A., Bustos, C. S., Chisti, Y., & Sevilla, J. M. F. (2010). Protein measurements of microalgal and cyanobacterial biomass. Bioresource technology, 101(19), 7587-7591.

Lourenço, S. O., Barbarino, E., De-Paula, J. C., Pereira, L. O. D. S., & Marquez, U. M. L. (2002). Amino acid composition, protein content and calculation of nitrogen-to-protein conversion factors for 19 tropical seaweeds. Phycological Research, 50(3), 233-241.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. Journal of biological chemistry, 193(1), 265-275.

Maddocks, S. E., & Oyston, P. C. (2008). Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins. Microbiology, 154(Pt 12), 3609-3623. doi:10.1099/mic.0.2008/022772-0

Malkin, R., & Niyogi, K. (2000). Biochemistry and molecular biology of plants. Rockville, MD, USA: American Society of Plant Physiologists, 568-628.

Mohtashamian, Marjan Sadat, "The Use of a Mixed *Chlorella* Cyanobacteria Culture as a Protein Source for Aquaculture" (2012). LSU Master's Theses. 4288. https://digitalcommons.lsu.edu/gradschool_theses/4288

Mossé, J. 1990. Nitrogen to protein conversion factor for ten cereals and six legumes or oilseeds. A reappraisal of its definition and determination. Variation according to species and to seed proteic content. J. Agric. Food Chem. 38: 18–24.

Nielsen S.S., (2010). Food Analysis Laboratory Manual, 2nd ed., Springer US, New York.

Price, C. A. (1965). A membrane method for determination of total protein in dilute algal suspensions. Analytical biochemistry, 12(2), 213-218.

Roy, H., & Cannon, S. (1988). Ribulose bisphosphate carboxylase assembly: what is the role of the large subunit binding protein. Trends in biochemical sciences, 13(5), 163-165.

Rypniewski, W. R., Breiter, D. R., Benning, M. M., Wesenberg, G., Oh, B. H., Markley, J. L., . . . Holden, H. M. (1991). Crystallization and structure determination of 2.5-.ANG. resolution of the oxidized iron-sulfur [2Fe-2S] ferredoxin isolated from Anabaena 7120. Biochemistry, 30(17), 4126-4131. doi:10.1021/bi00231a003

Safi, C., Charton, M., Ursu, A. V., Laroche, C., Zebib, B., Pontalier, P. Y., & Vaca-Garcia, C. (2014). Release of hydro-soluble microalgal proteins using mechanical and chemical treatments. Algal research, 3, 55-60.

Schägger, H., & Von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Analytical biochemistry, 166(2), 368-379.

Silaban, A. G. (2012). Growth rate and productivity of a Louisiana native microalgae/cyanobacteria co-culture: Feasibility for use in industrial biotechnology

applications. Louisiana State University Graduate School Digital Commons. Dept of Civil and Environmental Engineering. Master's Thesis.

Slocombe, S. P., Ross, M., Thomas, N., McNeill, S., & Stanley, M. S. (2013). A rapid and general method for measurement of protein in micro-algal biomass. Bioresource technology, 129, 51-57.

Stock, A. M., Robinson, V. L., & Goudreau, P. N. (2000). Two-Component Signal Transduction. Annual Review of Biochemistry, 69(1), 183-215. doi: 10.1146/annurev.biochem.69.1.183

Swanson, R. V., & Glazer, A. N. (1990). Separation of phycobiliprotein subunits by reverse-phase high-pressure liquid chromatography. Analytical Biochemistry, 188(2), 295-299. doi: https://doi.org/10.1016/0003-2697(90)90609-D

Tang, X. S., & Satoh, K. (1985). The oxygen-evolving photosystem II core complex. FEBS letters, 179(1), 60-64.

Ursu, A. V., Marcati, A., Sayd, T., Sante-Lhoutellier, V., Djelveh, G., & Michaud, P. (2014). Extraction, fractionation and functional properties of proteins from the microalgae *Chlorella vulgaris*. Bioresource technology, 157, 134-139.

Wydrzynski, T. J., & Satoh, K. (2005). Photosystem II: The light-driven water: plastoquinone oxidoreductase. Dordrecht: Springer.

Yeoh, H. H. and Truong, V. D. 1996. Protein contents, amino acid compositions and nitrogen-to-protein conversion factors for cassava roots. J. Sci. Food Agric. 70: 51–4.

Chapter 5. Carbohydrate and Starch Characterization of Louisiana Native Co-Culture of Microalgae (*Chlorella Vulgaris* L.) and Cyanobacteria (*Leptolyngbya* sp.)

5.1. Introduction

Microalgae is a robust source of functional ingredients with positive health effects due to high PUFAs, polysaccharides, pigments, essential minerals, vitamins, enzymes and bioactive peptides. Better understanding of the carbohydrates present in algae strains encourages use in food products, and food applications. Carbohydrate levels in *Arthrospira platensis* (16%) and *Chlorella* species (22%) have been previously determined by Kim and others (2015) but carbohydrate content varies based on algae culture type and growth conditions. There is limited info available on algal carbohydrate chemical properties in co-cultures. Polysaccharides from plants have applications in food, pharmaceutical, biofuel, bioethanol and biomedical industries because of their compatibility, biodegradability, and non-toxicity (Guzman and others, 2003; Vertes and others, 2008; Zhang and others, 2008; Trabelsi and others, 2009; Goo and others, 2013). Cell wall polysaccharides and starch can be converted into fermentable sugars for bioethanol production by microbial fermentation (Wang and others, 2011). Previous studies have described algae's bioactive compounds and suggested they be used to develop new drugs and health foods (Nagai & Yukimoto, 2003; Zhang and others, 2010).

Algae polysaccharides are made up of many monosaccharides connected with glycosidic bonds. These polysaccharides were researched by others and found to possess immunological properties like stimulating the immune system, being anti-tumor, anti-viral, having antioxidant and anti-mutagenic properties (Bohn and BeMiller, 1995; Kennedy and White, 1983; Kennedy, 1989; Zhang and others, 2010). The objective of this study was characterization of Louisiana native co-culture of microalgae (*Chlorella vulgaris* L.) and cyanobacteria (*Leptolyngbya* sp.)

(CCA) carbohydrates and starches, information that is imperative for application of these carbohydrates and starches in the future.

5.2. Materials/Experimental Design

Louisiana native co-culture of microalgae (*Chlorella vulgaris* L.) and cyanobacteria (*Leptolyngbya* sp.) (CCA) was provided by Dr. Gutierrez-Wing in the Aquatic Germplasm and Genetic Resources center of the School of Renewable Natural Resources, LSU Ag Center. The CCA was cultivated using the growth parameters: scalar irradiance, pH 7-9, temperature 25 ± 2°C, Bold 1NV growth media, and 40 LPM (liters per minute) aeration. Two irradiance treatments were applied to CCA in this study. CCA was cultivated in 6 cultures, 3 cultures were exposed to average scalar irradiance (ASI) 1041 ± 269.18 μmol m^{-2} s^{-1} PAR and will be referred to as Treatment 1 and the other 3 cultures were exposed to ASI 430 ± 96.03 μmol m^{-2} s^{-1} PAR and will be referred to as a Treatment 2 .

Algae was harvested during stationary growth phase which is determined as on the first day of decrease in optical density, using a semi-continuous flow centrifuge at 2L/min. All cultures were frozen after harvest and stored at 4°C short-term (2 months) or at -20˚C long-term (3-12 months).

The experiment was conducted in a completely randomized design (CRD), with no blocks. There were 6 tanks chosen at random to contain one of the two treatment levels, Treatment 1 – cultures exposed to ASI of 1041 ± 269.18 μmol m^{-2} s^{-1} PAR and Treatment 2– cultures exposed to ASI 430 ± 96.03 μmol m^{-2} s^{-1} PAR. The co-culture species ratio (*Chlorella*: Cyanobacteria) was the response of the treatments. The experimental and sampling units were the algae tanks.

Statistical analysis was performed using two-sample t-test (GraphPad, QuickCalc) available at https://www.graphpad.com/quickcalcs/ . The two-sample t-test was performed at 95% confidence to determine the difference in the two treatments, this test compared the average means of each dependent variable (assay performed).

Phenol sulfuric method gave total sugar content. Megazyme Starch kits provided resistant starch, total starch, and amylose/amylopectin ratio of CCA. DSC provided thermal properties of CCA starch. GC-MS assayed total monosaccharide content.

5.3. Carbohydrate Characterization Methods

5.3.1. Total Carbohydrate Content

The phenol-sulfuric acid method was used to find the total sugar content, the phenol-sulfuric acid method is a colorimetric method used to determine total classes of carbohydrates, including mono-, di-, oligo-, and polysaccharides in samples. Algae products are high in hexose sugars therefore glucose was used as a standard curve for this sample, at absorbance 490nm (Nielson, 2010).

5.3.1.1. Sample Preparation

Microalgae co-culture was defatted (lipid removed) with ethanol (Li and others, 2014). Defatting was also done with the Bligh and Dyer (1959) method, before acquiring the total monosaccharide content. After defatting, algal biomass was hydrolyzed in a two-stages: one hour at 30°C in 72% sulfuric acid, then one hour at 121°C in 4% sulfuric acid (Templeton and others, 2012). After hydrolysis, the insoluble residue was filtered and separated from the hydrolysate (Templeton and others, 2012).

5.3.1.2. Phenol-Sulfuric Methodology

Glucose standard serial dilutions and the prepped algae samples tested contained 20–100 mg glucose/2 ml. Defatted algae samples were placed into separate Eppendorf tubes, 300 µL of stock H_2SO_4 was added, then 60 µL of 5% phenol was added. Tubes were placed in a 90˚C water bath for 5 minutes. All standard and samples were transferred to a microplate in triplicate and read at 490 nm with 0 µg glucose/2 mL read as the blank (Neilson, 2010).

5.3.2. Identifying Monosaccharides by GC-MS

5.3.2.1. Sample Preparation

Algae samples (10mg) were hydrolyzed in 0.9 mL of 2N TFA and incubated for 1 hour at 121˚C, samples were cooled and spiked with internal standard myo-inositol at 100ppm concentration. Hydrolysates were filtered through a PES filter, and the TFA was evaporated off using a vacuum evaporator at 80˚C. To reduce monosaccharides to alditols, dried hydrolysates were dissolved in 1 mL of 2% sodium borohydride solution, then heated to 40˚C for 90 min. After reduction and cooling to room temperature, 100 uL of glacial acetic acid was added to decompose sodium borohydride. To acetylate alditols, 0.2 mL of 1-methylimidazole (catalyst) and 2 mL of acetic anhydride (acetylator) were added to the samples. Five mL of DI water was added to decompose acetic anhydride (Templeton and others, 2012). Then 1 mL of dichloromethane (DCM) was added, vortexed, then centrifuged (10 min 4500 rpm). Water was the supernatant and was removed using a Pasteur pipette, the lower DCM and monosaccharide layer was collected for GC analysis, this solution was diluted, if necessary, depending on the sensitivity of the GC-MS used (Koh and others, 2018).

5.3.2.2. System Conditions

DCM monosaccharide algae samples (3 uL) were run on the Thermo Scientific Trace GC Ultra, TriPlus Autosampler, and TSQ Quantum XLS System (Waltham, MA). Program details were as follows: oven initial temp 160°C increasing by 4 deg/min to 250°C, the max temp was set at 350°C, the run time was 50 min. The following settings were used, split flow was set to 10 mL/min, solvent valve temperature was 100°C, surge pressure was 0.50 psi, transfer rate: 14.5 deg/sec, transfer temp: 200°C, inject time: 0.5 min, transfer time: 1 min (Koh and others, 2018).

5.4. Starch Characterization Methods

5.4.1. Total Starch Content

The Megazyme total starch HK analysis procedure was used to quantify total starch in defatted algae samples. Starch was solubilized by incubating the lipid and protein removed freeze dried algae samples at 100°C with thermostable α-amylase. Thermostable α-amylase hydrolyzed starch into soluble branched and unbranched maltodextrins (3,000 U/mL α-amylase, pH 5.0, 100°C) (Megazyme, 2017; Neilson, 2010). Resistant starch in the sample was dissolved by mixing the sample with 2 M KOH at 4°C, followed by neutralization with sodium acetate buffer and hydrolysis with α-amylase. According to Megazyme, (2017) and McCleary and others (1997), amyloglucosidase broke maltodextrins down to D-glucose. D-Glucose was phosphorylated by the enzyme hexokinase and adenosine-5’-triphosphate to glucose-6-phosphate (G-6-P) forming adenosine-5’-diphosphate. In the presence of the enzyme G-6-P was oxidized by nicotinamide-adenine dinucleotide phosphate (NADP+) to gluconate-6-phosphate with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH). The amount of NADPH formed in this reaction was stoichiometric to the amount of D-glucose. NADPH was

measured by the increase in absorbance at 340 nm (McCleary and others, 1997; Megazyme, 2017).

5.4.2. Resistant Starch Content

Resistant starch content of all lipid and protein removed freeze dried algae samples was determined by the Megazyme procedure (Megazyme International Ireland Limited, Bray, Ireland). This method is approved by AOAC method 2002.02 and AACC method 32-40. All reagents and enzymes used were analytical grade. Absorbances of all samples were read at 510 nm against the reagent blank. This method allowed the measurement of resistant starch, solubilized starch, and total starch content of samples. Samples were incubated in a stirring water bath with pancreatic α-amylase and amyloglucosidase (AMG) for 16 h at 37°C, non-resistant starch was solubilized and hydrolyzed to D-glucose by the enzymes. This reaction was stopped by adding an equal volume of ethanol, the resistant starch was recovered as a pellet after centrifugation. This pellet was washed two times with 50% ethanol, then centrifuged again, the supernatant was removed. Resistant starch in the pellet was then dissolved in 2 M KOH while stirring in an ice-water bath. This solution was neutralized with acetate buffer and the starch was further hydrolyzed to D-Glucose with AMG. D-Glucose was measured with glucose oxidase/peroxidase reagent (GOPOD) which was a measure of the resistant starch content of the sample. Non-resistant starch (solubilized starch) was determined by combining the supernatants, adjusting the volume, then measuring D-glucose content with GOPOD (Megazyme, 2015).

5.4.3. Amylose/Amylopectin Content

Lipid and protein removed freeze dried algae samples were dissolved by heating in dimethyl sulfoxide (DMSO). Lipids were removed by precipitating the starch in ethanol and collecting the starch precipitate. After dissolving the sample precipitate in an acetate or salt

solution, amylopectin was precipitated by the addition of lectin concanavalin A (Con A) then isolated by centrifugation. The amylose (supernatant) was hydrolyzed by an enzyme to D-glucose, which was then evaluated using glucose oxidase/peroxidase reagent (GOPOD). The total starch, in a separate aliquot of the acetate/salt solution, was hydrolyzed to D-glucose and measured colorimetrically by GOPOD. The concentration of amylose in the starch sample was the ratio of GOPOD absorbance at 510 nm of the supernatant of the Con A sample after precipitation to that of the total starch sample (Megazyme, 2016). Amylose/amylopectin ratios effect gelation, solubility, and resistant starch content. Starches with high levels of branched chains are less likely to crystallize after processing (Chang, 2012). The ratio and structure of these components affect how digestible starch is. High-amylose starch is more resistant to digestion than low-amylose starch (Khawas and Deka, 2017). The Megazyme amylose/amylopectin method is a modified version of the Con A method developed by Yun and Matheson (1990).

5.4.4. Thermal Properties of CCA Starch

To purify starch, freeze dried algae (0.5 g) was resuspended in 10 mL of 10 mM Tris acetate, pH 7.5, 1 mM EDTA. Algal suspensions were disrupted by sonication for 15 min with 10s pulse/rest intervals on ice. A crude starch extract was obtained by centrifuging the lysate (sonicated cells) (10,000g for 15 min). The pellet obtained was resuspended in 1 mL of 90% Percoll. The gradient was formed by centrifugation (10,000g for 30 min) this was done 2 times. The purified starch pellet was rinsed in DI water, centrifuged (10,000g for 10 min), and kept dry at 4°C (Ball and others, 1991; Delrue and others., 1992; Zeeman and others, 1998; Ral and others 2004).

To separate starch polysaccharides, 10mg of starch was solubilized in 0.5 ml DMSO at 100ºC for 30 min, 99.5ml of DI water was added. This solution was eluted with 0.01M NaOH at a flow rate of 10ml/min on a Sepharose CL2B column (1.6 x 145 cm), 3ml fractions were collected and lyophilized (Izumo and others, 2007; Shi and others, 2007).

Isolated algae starches were analyzed for thermal properties using a differential scanning calorimeter (DSC) (TA Q100, TA Instruments, Newcastle, DE) in duplicate. Ten mg of isolated algae starch was weighed into a steel high volume DSC pan. Then 20 µl of DI water was placed into the starch in the DSC pan. The pan was sealed and stored at 25°C overnight to allow starch hydration creating a starch paste. The pans were heated in the DSC from 25 °C to 140 °C at a rate of 5 °C /min. Another pan containing 20 µl DI water was used as a reference to compare heat capacity. The run time was 30 mins per sample. The thermal transition parameters, including change in enthalpy (J/g), onset temperature and peak temperature were determined using Universal Analysis 2000 (TA Q100, TA Instruments, Newcastle, DE) (Jiang, 2013). The DSC was calibrated using an indium standard.

5.5. Results and Discussion

5.5.1. Total Sugar Content by Phenol Sulfuric Method

Total sugar content was calculated as 25.44±6.90g /100g of CCA for treatment 1, and 19.28±2.84g /100g of CCA for treatment 2 (Table 5.1.). There was no significant difference at 95% confidence between treatments (p=0.10). These findings coincide with previous studies finding where sugars were calculated as 20-25% of *Leptolyngbya* sp. KIOST-1 and *Chorella* (DWB) (Kim and others, 2015; Kumar and others, 2016).

Table 5.1 Average Total Sugar Content

	Avg Carb (g sugar/100g of algae DWB)
Treatment 1	25.44 ± 6.90 a
Treatment 2	19.28 ± 2.84 a

Like lettering represents no significant difference among treatments at 95% confidence. Treatment 1 average scalar irradiance of 1041 ± 269.18 μmol m-2 s-1 PAR. Treatment 2 ASI of 430 ± 96.03 μmol m-2 s-1 PAR. n = 6.

5.5.2. Identifying Monosaccharides by GC-MS

Total monosaccharide content for treatment 1 was identified as 1.36 ± 0.11g per 100 g of algae DWB, and treatment 1 was 1.44 ± 0.09 g per 100 g of algae DWB. Table 5.2. displays the monosaccharide composition for both treatments. Mannose, Glucose and Galactose were the most prevalent monosaccharides in CCA. These monosaccharides were identified and quantified by GC-MS standard curve quantification with monosaccharide standards. There was no significant difference between treatments 1 and 2 for total monosaccharides at 95% confidence (p=0.101). None of the individual monosaccharides were significantly different across treatments except for fucose that was significantly higher in treatment 1 (p=0.23). Ho and others (2013) studied *C. vulgaris* and noted that glucose was the dominant sugar component in microalgae-based carbohydrates, they found the rest of the sugar components were xylose, galactose, arabinose, and rhamnose. Kim and others (2015) studied Cyanobacteria *Leptolyngbya* sp. KIOST-1 and noted that glucose was the dominant sugar, followed by galactose, mannose, and fucose, rhamnose, xylose, and arabinose.

Table 5.2 Average Monosaccharide Content

Monosaccharide	Treatment 1		Treatment 2	
	Content (g/100g algae)	Composition (% TM)	Content (g/100g algae)	Composition (% TM)
Mannose	0.53 ± 0.04a	39	0.58 ± 0.08a	40
Glucose	0.49 ± 0.04a	36	0.50 ± 0.04a	35
Galactose	0.20 ± 0.02a	15	0.22 ± 0.02a	15
Fucose	0.08 ± 0.03a	6	0.05 ± 0.02b	3
Rhamnose	0.03 ± 0.01a	2	0.03 ± 0.01a	2
Arabinose	0.02 ± 0.01a	2	0.04 ± 0.01a	3
Xylose	0.01 ± 0.01a	1	0.01 ± 0.01a	1
Total Monosaccharides (TM)	1.36 ± 0.11a	100	1.44 ± 0.09a	100

Like lettering represents no significant difference among treatments at 95% confidence. Treatment 1 average scalar irradiance of 1041 ± 269.18 μmol m-2 s-1 PAR. Treatment 2 ASI of 430 ± 96.03 μmol m-2 s-1 PAR. n = 6.

5.5.3. Starch Characterization

Megazyme Starch Kits provided the amount of total, resistant, non-resistant, and amylose/amylopectin starch present in CCA. Total starch (TS) was 17.95 ± 2.72 g TS/100 g of algae DWB for treatment 1 and 15.75 ± 4.27 g TS/100 g of algae DWB for treatment 2, there was no significant difference among treatments at 95% confidence (p=0.71) (Table 5.3.).

Table 5.3. Total Starch in CCA

	Avg (g starch/ 100 g algae DWB)
Treatment 1	17.95 ± 2.72 a
Treatment 2	15.75 ± 4.27 a

Like lettering represents no significant difference among treatments at 95% confidence. Treatment 1 average scalar irradiance of 1041 ± 269.18 μmol m-2 s-1 PAR. Treatment 2 ASI of 430 ± 96.03 μmol m-2 s-1 PAR. n = 6.

Non-resistant starch (NRS) was 12.96 ± 2.73 g NRS/100 g of algae DWB for treatment 1 and 11.37 ± 0.72 g NRS/100 g of algae DWB for treatment 2, there was no significant difference among treatments at 95% confidence (p=0.18). Quantified resistant starch (RS) content was 5.79 ± 2.73 g RS /100 g of algae for treatment 1 and 5.02 ± 4.37 g RS /100 g of algae DWB for treatment 2, there was no significant difference among treatments at 95% confidence (p=0.23). Total sugar content was calculated as 25.44 ± 6.90 g/ 100 algae DWB for treatment 1. It can be

concluded that 71% of Treatment 1 CCA's carbohydrates are starch, comprised of 23% resistant starch, and 48% non-resistant starch. Total carbohydrate content for treatment 2 was 19.28±2.84 g/ 100 g of algae DWB, 82% of treatment 2's carbohydrates are starch, comprised of 26% resistant starch, and 56% non-resistant starch. The unaccounted carbohydrates are proposed as unextracted cell wall carbohydrates and cellulose (Chen and others, 2017; Al Abdallah and others, 2016).

Table 5.4. Non-Resistant Starch in CCA

	Avg (g non-resistant starch/ 100 g algae DWB)
Treatment 1	12.96 ± 2.73 a
Treatment 2	11.37 ± 0.72 a

Like lettering represents no significant difference among treatments at 95% confidence. Treatment 1 average scalar irradiance of 1041 ± 269.18 μmol m-2 s-1 PAR. Treatment 2 ASI of 430 ± 96.03 μmol m-2 s-1 PAR. n = 6.

Table 5.5. Resistant Starch in CCA

	Avg (g resistant starch/ 100 g algae DWB)
Treatment 1	5.79 ± 2.73 a
Treatment 2	5.02 ± 4.37 a

Like lettering represents no significant difference among treatments at 95% confidence. Treatment 1 average scalar irradiance of 1041 ± 269.18 μmol m-2 s-1 PAR. Treatment 2 ASI of 430 ± 96.03 μmol m-2 s-1 PAR. n = 6.

Amylose/Amylopectin Kit provided CCA starch characteristics; amylose content was 71.62 ± 7.18% w/w; amylopectin content was 28.28 ± 7.18% w/w for treatment 1 (Table 5.6.). Amylose content was 65.85 ± 3.87% w/w; amylopectin content was 34.15 ± 3.87 % w/w for treatment 2, there was no significant difference among treatments at 95% confidence (p=0.09). These results are conflicting with results of Gifuni and others (2017) who found *C. sorokiniana* starch to be 17% amylose and 83% amylopectin. This could be due to differences in starch extraction, and starch purity, for this analysis n= 8.The high amylose content of CCA is considered high according to Juliano (1971) who defined 25-30% amylose as high, 20-25% amylose as intermediate, 10-20% amylose as low, 2-9% amylose as very low, and 1-2% amylose as waxy. CCA starch could provide enhanced sensory effects such as crispiness, and gelling textures

(Chang, 2012). High-amylose starch is known to be more resistant to digestion than low-amylose starch (Birt and others, 2013). Starches that bypass digestion in the intestine and stomach are associated with health benefits like feeding the gut-healthy bacteria in the intestine and increasing production of short-chain fatty acids like butyrate (Morrison and Preston, 2016).

Table 5.6. Amylose/Amylopectin content in CCA Starch

	Amylose% (w/w)	Amylopectin% (w/w)
Treatment 1	71.62 ± 7.18 a	28.28 ± 7.18 a
Treatment 2	65.85 ± 3.87 a	34.15 ± 3.87 a

Like lettering represents no significant difference among treatments at 95% confidence. Treatment 1 average scalar irradiance of 1041 ± 269.18 μmol m-2 s-1 PAR. Treatment 2 ASI of 430 ± 96.03 μmol m-2 s-1 PAR. n = 6.

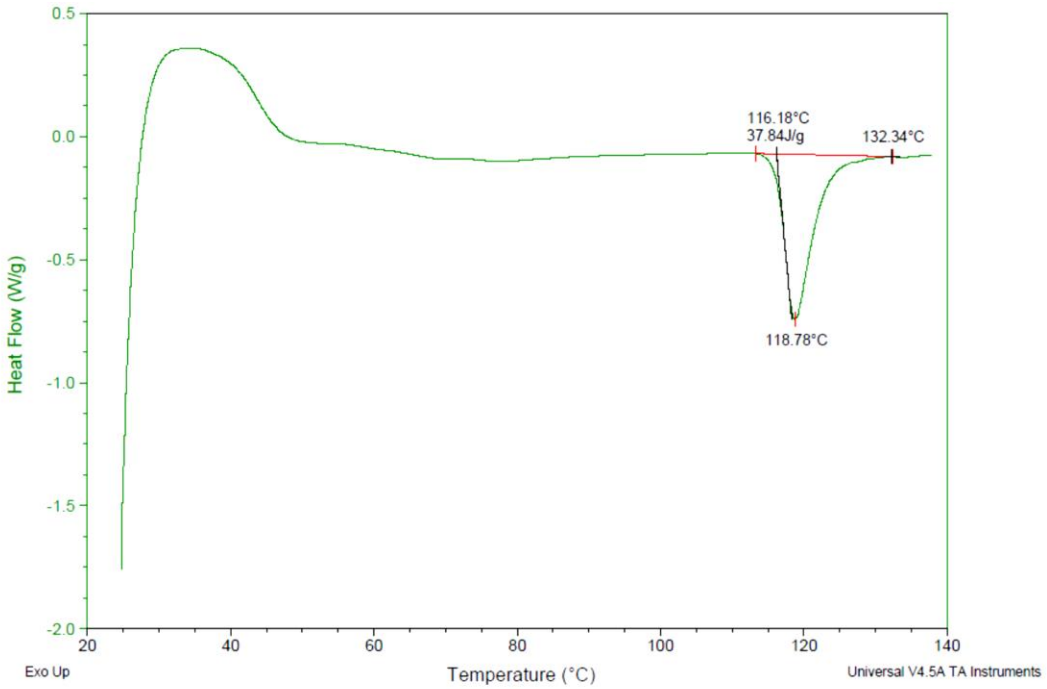
5.5.3.1. Thermal Properties of CCA Starch

DSC provided physicochemical characteristics of CCA starch. Gelatinization temperature was assessed (Izumo and others, 2007, Gill and others, 2010, Gifuni and others, 2017). The peaks in both treatments ~ 120 ºC indicate pasting of algae starch and the beginning of gelatinization. Due to the high temperature of the peaks (about 118.5 ºC) they may represent resistant starch which usually has a peak around 120 ºC, the onset temperature of 115 ºC indicates the swelling of the algae starch granules with water to create a gel (Figure 5.1-5.2.).

Table 5.7. Thermal Properties in CCA by DSC

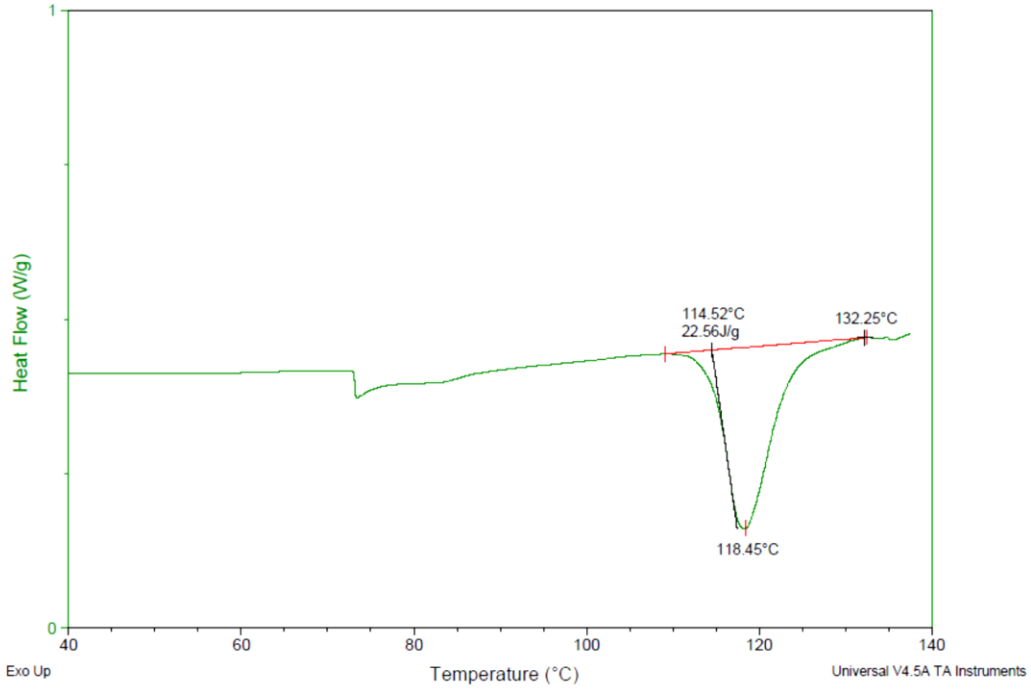
	Treatment 1	Treatment 2
Onset Temperature (ºC) T_O	116.18	114.52
Peak Temperature (ºC)T_P	118.78	118.45
Peak Enthalpy (J/g)	37.84	22.56
Conclusion Temperature (ºC)T_C	132.34	132.25
T_C -T_O (ºC)	16.16	17.73

Treatment 1 average scalar irradiance of 1041 ± 269.18 μmol m-2 s-1 PAR. Treatment 2 ASI of 430 ± 96.03 μmol m-2 s-1 PAR. n=2



Treatment 1 average scalar irradiance of 1041 ± 269.18 µmol m-2 s-1 PAR. Treatment 2 ASI of 430 ± 96.03 µmol m-2 s-1 PAR. n=2. Heat flow (W/g) vs. Temperature (ºC)

Figure 5.1. DSC Curve for CCA Treatment 1



Treatment 1 average scalar irradiance of 1041 ± 269.18 µmol m-2 s-1 PAR. Treatment 2 ASI of 430 ± 96.03 µmol m-2 s-1 PAR. n=2. Heat flow (W/g) vs. Temperature (ºC)

Figure 5.2. DSC Curve for CCA Treatment 2

5.6. Conclusion

Carbohydrates are the second largest macronutrient in CCA. The phenol sulfuric method indicated total sugar content as 25.44±6.90g/ 100g of CCA for treatment 1, and 19.28±2.84g/100g of CCA for treatment 2. Seven monosaccharides were identified and quantified from CCA, the greatest of which were mannose, glucose and galactose. Total monosaccharide content for treatment 1 was identified as 1.36 ± 0.11g per 100 g of CCA, and treatment 2 was 1.44 ± 0.09 g per 100 g of CCA. Amylose/amylopectin results conflicted with previous trends that found that green algae (*Chlorella*) species synthesize polysaccharides that are like amylopectin (Markou and others., 2012). Total sugar content was calculated as 25.44±6.90g/ 100g of CCA for treatment 1 (71% of Treatment 1 CCA's carbohydrates are starch, comprised of 23% resistant starch, and 48% non-resistant starch). Total sugar content for treatment 2 was 19.28±2.84g/100g of CCA (82% of treatment 2's carbohydrates are starch, comprised of 26% resistant starch, and 56% non-resistant starch). DSC results indicated CCA extracted starch had an increased thermodynamic range when compared to corn starch as its peaks at around 120ºC, indicating resistant starch presence. There was no significant difference found between treatments 1 and 2 for all carbohydrate analysis, this indicated that the CCA growing parameters (irradiance) can vary without significantly changing the carbohydrate produced in CCA. These co-culture algal carbohydrates could possibly be used as a source of "green" or vegan carbohydrates and nutraceuticals.

5.7. References

Ball S, Marianne T, Dirick L, Fresnoy M, Delrue B, Decq AA (1991). Chlamydomonas reinhardtii low-starch mutant is defective for 3-phosphoglycerate activation and orthophosphate inhibition of ADP-glucose pyrophosphorylase. Planta 185: 17–26.

Birt D., Boylston, T., Hendrich, S., Jane, J., Hollis, J., Li, L., McClelland, J., Moore, S., Phillips, G., Rowling, M., Schalinske, K., Scott, M., Whitley, E. (2013). Resistant Starch: Promise for Improving Human Health. Advances in Nutrition, 4, 587-601.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. Canadian journal of biochemistry and physiology, 37(8), 911-917.

Chang, Chuan. (2012). Carbohydrates- Comprehensive Studies on Glycobiology and Glycotechnology. InTech. 290-316.

Delrue B, Fontaine T, Routier F, Decq A, Wieruszeski J-M, Van den Koornhuyse N, Maddelein ML, Fournet B, Ball S (1992) Waxy Chlamydomonas reinhardtii: monocellular algal mutants defective in amylose biosynthesis and granule-bound starch synthase activity accumulate a structurally modified amylopectin. J Bacteriol 174: 3612–3620.

Domozych, D., Ciancia, M., Fangel, J. U., Mikkelsen, M. D., Ulvskov, P., & Willats, W. G. (2012). The cell walls of green algae: a journey through evolution and diversity. Frontiers in plant science, 3, 82.

Gifuni, I., Olivieri, G., Krauss, I. R., D'Errico, G., Pollio, A., & Marzocchella, A. (2017). Microalgae as new sources of starch: isolation and characterization of microalgal starch granules. Chemical Engineering Transactions, 57, 1423-1428.

Gill, P., Tohidu Moghadam, T., Ranjbar, B. (2010). Differential Scanning Calorimetry Techniques: Applications in Biology and Nanoscience. Biomolecular Techniques. 21, 167-193.

Goo, B. G., Baek, G., Choi, D. J., Park, Y. I., Synytsya, A., Bleha, R., ... & Park, J. K. (2013). Characterization of a renewable extracellular polysaccharide from defatted microalgae Dunaliella tertiolecta. Bioresource technology, 129, 343-350.

Guzman, S., Gato, A., Lamela, M., Freire-Garabal, M., & Calleja, J. M. (2003). Anti-inflammatory and immunomodulatory activities of polysaccharide from *Chlorella* stigmatophora and Phaeodactylum tricornutum. Phytotherapy Research, 17(6), 665-670.

Hall, N. G., & Schönfeldt, H. C. (2013). Total nitrogen vs. amino-acid profile as indicator of protein content of beef. Food Chemistry, 140(3), 608-612. doi: https://doi.org/10.1016/j.foodchem.2012.08.046

Ho, S.-H., Huang, S.-W., Chen, C.-Y., Hasunuma, T., Kondo, A., & Chang, J.-S. (2013). Characterization and optimization of carbohydrate production from an indigenous microalga *Chlorella vulgaris* FSP-E. Bioresource Technology, 135(Supplement C), 157-165. doi: https://doi.org/10.1016/j.biortech.2012.10.100

Jiang, Yu, "Effects of Amino Acids and Fatty Acids on Rice Starch Properties: Thermal, Pasting, Resistant Starch and Structural Characterization" (2013). LSU Doctoral Dissertations. 1943. https://digitalcommons.lsu.edu/gradschool_dissertations/1943

Juliano, B. (1971). A simplified assay for milled-rice amylose. Cereal Sci. Today, 16, 334-360.

Karkacier, M., Erbas, M., Uslu, M. K., & Aksu, M. (2003). Comparison of different extraction and detection methods for sugars using amino-bonded phase HPLC. Journal of chromatographic science, 41(6), 331-333.

Kim, J. H., Choi, W., Jeon, S.-M., Kim, T., Park, A., Kim, J., . . . Kang, D.-H. (2015). Isolation and characterization of *Leptolyngbya* sp. KIOST-1, a basophilic and euryhaline filamentous cyanobacterium from an open paddle-wheel raceway Arthrospira culture pond in Korea. Journal of Applied Microbiology, 119(6), 1597-1612. doi:10.1111/jam.12961

Koh, J., Xu, Z. and Wicker, L. (2018), Blueberry Pectin Extraction Methods Influence Physico-Chemical Properties. Journal of Food Science, 83: 2954-2962. doi:10.1111/1750-3841.14380

Li, Y., Ghasemi Naghdi, F., Garg, S., Adarme-Vega, T. C., Thurecht, K. J., Ghafor, W. A., … Schenk, P. M. (2014). A comparative study: the impact of different lipid extraction methods on current microalgal lipid research. Microbial Cell Factories, 13, 14. http://doi.org/10.1186/1475-2859-13-14

Monsur, H. A., Jaswir, I., Simsek, S., Amid, A., & Alam, Z. (2017). Chemical structure of sulfated polysaccharides from brown seaweed (Turbinaria turbinata). International Journal of Food Properties, 20(7), 1457-1469. doi:10.1080/10942912.2016.1211144

Morrison, D. J., & Preston, T. (). Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut microbes, 7(3), 189–200. doi:10.1080/19490976.2015.1134082

Nielsen S.S., (2010). Food Analysis Laboratory Manual, 2nd ed., Springer US, New York.

Ortiz-Tena, J. G., Rühmann, B., Schieder, D., & Sieber, V. (2016). Revealing the diversity of algal monosaccharides: Fast carbohydrate fingerprinting of microalgae using crude biomass and showcasing sugar distribution in *Chlorella vulgaris* by biomass fractionation. Algal Research, 17, 227-235. doi: https://doi.org/10.1016/j.algal.2016.05.008

Ral, J.-P., Derelle, E., Ferraz, C., Wattebled, F., Farinas, B., Corellou, F., . . . Ball, S. (2004). Starch Division and Partitioning. A Mechanism for Granule Propagation and Maintenance in the Picophytoplanktonic Green Alga <em>Ostreococcus tauri</em&gt. Plant Physiology, 136(2), 3333. doi:10.1104/pp.104.044131

Ringuette, C. The Development of a Reduced Glycemic Load/High Fiber Pasta Using Pulses. (2016). LSU Master's Theses. 2707. https://digitalcommons.lsu.edu/gradschool_theses/2707

Rioux, L. E., Turgeon, S. L., & Beaulieu, M. (2007). Characterization of polysaccharides extracted from brown seaweeds. Carbohydrate polymers, 69(3), 530-537.

Shi, Y. J. Yang, S.F. Hu, Q. (2007). Purification and identification of polysaccharide derived from *Chlorella* pyrenoidosa. Food Chemistry, 103 (2007) 101–105. doi:10.1016/j.foodchem.2006.07.028

Sichina, W. J. (2000). Use of DSC for the Characterization of Starches. PerkinElmer Instruments.

Trabelsi L, M'sakni NH, Ben Ouada H, Bacha H, Roudesli S. 2009. Partial characterization of extracellular polysaccharides from the CyanobacteriumArthrospira platensis. Biotech Biop Eng.;14:27–31.

Vertes, A.A., Inui, M., Yukawa, H., 2008. Technological options for biological fuel ethanol. J. Mol. Microbiol. Biotechnol. 15, 16–30.

Wang, X., Liu, X.H., Wang, G.Y., 2011. Two-stage hydrolysis of invasive algal feedstock for ethanol fermentation. J. Integr. Plant. Biol. 53 (3),246–252.

Zeeman S, Northrop F, Smith AM, ap Rees T (1998) A starch-accumulating mutant of Arabidopsis thaliana deficient in a chloroplastic starch hydrolyzing enzyme. Plant J 15: 357–365.

Chapter 6. Lipid Characterization of Louisiana Native Co-Culture of Microalgae (*Chlorella Vulgaris* L.) and Cyanobacteria (*Leptolyngbya* sp.)

6.1. Introduction

Microalgae contain triacylglycerol and membrane lipids (Araki, 1991; Harwood, 1998; Aakanksha and others, 2010; Bai, 2012). Algal lipids serve as energy reserves in the microalgal cell (Nakamura and Li-Beisson, 2016). Microalgal and biofuel industries, are interested in using the high value market lipids (Halim and others, 2012). Microalgae has gained commercial industrial attention due to its ability to synthesize high levels of long-chain PUFAs (14 or more carbons) when exposed to environmental factors such as stress (Yao and others, 2015). The composition and fatty acid profile of lipids extracted from algae is affected by the cultivation methods, temperature, irradiance, ratio of light/dark cycle and, aeration rate. During photosynthesis microalgae accumulate neutral lipids as triacylglycerols, these triacylglycerols can then be trans-esterified into fatty acid methyl esters (Zhu and others, 2016). The lipid content of microalgae varies for different species. This study's objective is to characterize total lipids, and fatty acids in *Chlorella vulgaris* (Chlorophyta)/*Leptolyngbya* sp. (Cyanobacteria) co-culture (CCA).

6.2. Materials/Experimental Design

Louisiana native co-culture of microalgae (*Chlorella vulgaris* L.) and cyanobacteria (*Leptolyngbya* sp.) (CCA) was provided by Dr. Gutierrez-Wing in the Aquatic Germplasm and Genetic Resources center of the School of Renewable Natural Resources, LSU Ag Center. The CCA was cultivated using the growth parameters: scalar irradiance, pH 7-9, temperature 25 ± 2°C, Bold 1NV growth media, and 40 LPM (liters per minute) aeration. Two irradiance treatments were applied to CCA in this study. CCA was cultivated in 6 cultures, 3 cultures were

exposed to average scalar irradiance (ASI) 1041 ± 269.18 μmol m^{-2} s^{-1} PAR and will be referred to as Treatment 1 and the other 3 cultures were exposed to ASI 430 ± 96.03 μmol m^{-2} s^{-1} PAR and will be referred to as a Treatment 2 .

Algae was harvested during stationary growth phase which is determined as on the first day of decrease in optical density, using a semi-continuous flow centrifuge at 2L/min. All cultures were frozen after harvest and stored at 4°C short-term (2 months) or at -20˚C long-term (3-12 months).

The experiment was conducted in a completely randomized design (CRD), with no blocks. There were 6 tanks chosen at random to contain one of the two treatment levels, Treatment 1 – cultures exposed to ASI of 1041 ± 269.18 μmol m^{-2} s^{-1} PAR and Treatment 2– cultures exposed to ASI 430 ± 96.03 μmol m^{-2} s^{-1} PAR. The co-culture species ratio (*Chlorella*: Cyanobacteria) was the response of the treatments. The experimental and sampling units were the algae tanks.

Statistical analysis was performed using two-sample t-test (GraphPad, QuickCalc) available at https://www.graphpad.com/quickcalcs/ . The two-sample t-test was performed at 95% confidence to determine the difference in the two treatments, this test compared the average means of each dependent variable (assay performed). Bligh Dyer, fat hydrolysis and hexane extraction by ASE provided total lipid content. GC-FID provided the fatty acid profile of CCA.

6.3. Lipid Characterization Methods

6.3.1. Sample Preparation by Modified Bligh Dyer Method

The Bligh and Dyer method is the standard method for the determination of total lipids in biological compounds. Freeze-dried algae (100 mg) was extracted in 10 mL of 2:1:1 methanol,

chloroform and water. This sample was freeze thawed three times (-20℃ for 4 h, then room temp for 2 h to lyse cells), this solution was shaken at 110 RPM for 20 mins and centrifuged 10 min 4000 RPM) after phase separation, lipids were collected/quantified in the chloroform phase, chloroform was evaporated off using a nitrogen stream (Bligh and Dyer, 1959, Halim and others, 2012).

6.3.2. Total Lipid Content Methods

6.3.2.1. Modified Bligh Dyer Method

The above sample preparation method was used to quantify total lipids by using equation 6.1 from Bligh and Dyer (1959) where the volume of the chloroform layer (mL) is subtracted from the weight of liquid aliquot (mg) then divided by the volume of the aliquot (mL) Total lipid is expressed as mg/g of algae then converted to g/ 100 g of algae.

Equation 6.1 Total Fat Equation

$$\text{Total Lipid} = \frac{(\text{Weight of Lipid Aliquot} - \text{Volume of Chloroform Layer})}{\text{Volume of Aliquot}}$$

6.3.2.2. Fat Hydrolysis by Soxhlet Apparatus

Method AOAC 922.06 was used to determine fat content by fat hydrolysis. Three grams of lyophilized algae sample was weighed and placed in a beaker. Ten grams of celite (SiO_2) was added and the weight was recorded. This mixture was filtered (HYDROTHERM C. Gerhardt #1004092) into a collection funnel then refluxed with 15% hydrochloric acid (w/v). Samples were dried for 30 minutes at 100°C then placed into cellulose thimble for extraction. The initial beaker weights and thimbles weights were recorded. Thimbles were placed in corresponding beakers and 90 mL of petroleum ether was added to the beaker and samples were extracted on the Soxhlet apparatus (SOXTHERM) (Königswinter, Germany). After 3 h, thimbles were

removed, and the beakers were dried for 30 minutes at 100°C. The final beaker weights were recorded.

6.3.2.3. Non-Polar Fat Content by ASE

Three grams of freeze-dried algae was mixed with 10 g of diatomaceous earth (activated charcoal) and packed into an extraction receptacle topped off with diatomaceous earth. This sample was weighed then refluxed on a Dionex ASE 350 (Thermo Scientific) with hexane extraction temperature range of 75-125 ℃, extraction time 15 min, extraction cycle was run 3 times. Extraction parameters were as follows set as 50% of flush volume and 80s of purge time for microalgal lipid extraction. The final microalgal lipid quantity extracted for ASE operation was expressed as lipid % based on algal dry weight. mass of lipid remaining is measured (M_{lipid}). The percentage of lipid in the initial sample (M_{sample}) can then be calculated:

Equation 6.2 Total Lipid Percent

$$\%\text{Lipid} = \frac{M_{\text{lipid}}}{M_{\text{sample}}} x\ 100$$

6.2.3.3. Fatty Acid Profile by GC-FID

One gram of lyophilized CCA was lipid extracted in 20mL of chloroform: methanol (2:1 v/v) for 1 h, shaken at 110 RPM. The methanol was extracted of using a nitrogen stream. About 10 mL of lipid chloroform extract was ascertained. Aliquots of this oil extract was used for FAME assay.

Official method AOCS Ce 2-66vwas used to prepare methyl esters using the following protocol: 500 milligrams of CCA lipid extract was added to 8 mL of 0.5 N Methanolic sodium hydroxide 12%, in a boiling flask this was refluxed 5 min. Nine milliliters of 12% v/v Boron trifluoride BF^3 – in methanol was added through the condenser and further boiled 2 min. Five ml

of hexane was added through the condenser and boiled 1 min. Boiling was stopped and 15 ml of saturated NaCl was added to the flask which stoppered and vortexed. Saturated NaCl was used to float the lipid to the top of the flask where it was extracted by Pasteur pipette. , (Petkov and Garcia, 2007). After fatty acids were esterified into methyl esters the internal standard (1ml of 10mg/ml methyl tridecanoate in hexane) was added (Petkov and Garcia, 2007; Van Wychen, and Laurens, 2013). One hundred microliters of the upper FAME containing layer was assayed by gas chromatography (GC) using an Agilent 7820 GC with a 30 m long Supelcowax-10 capillary column at 95 °C and flame ionization detector (FID). FID settings were 280°C, 450 mL/min zero air, 40 mL/min H2, 30 mL/min helium. This method was adapted from AOAC Method 969.33 modified with Petkov and Garcia's (2007) and Van Wychen, and Laurens (2013) protocol for microalgae. Fatty acids were identified using GLC reference substances. FAME were expressed as % in total fatty acids of CCA of total fat, then % FA in Total FA (w/w).

6.4. Results and Discussion

6.4.1. Total Lipid Content

Total lipid gravimetric analyses were extracted in organic solvents that removed hydrophilic cellular components like carbohydrates, and proteins. Extracted lipids in organic solvents were dried down with nitrogen gas. An estimation of total lipid content was determined by dry weight accounting for the weight of all solvent soluble components (fatty acids, glycerolipids, sphingolipids, triterpenoids and chlorophyll or other pigments, as well as non-lipid contaminants) (Grima and others, 1994). Total lipid content by Bligh Dyer extraction yield the lowest lipid contents (0.47 and 0.74g fat/ 100g algae DWB for treatment 1 and 2 respectively), while hexane extraction with Accelerated Solid Extraction (ASE) had the highest total lipid yield for CCA (8.20 ± 1.20 and 12.70 ± 2.50g/ 100g algae for treatment 1 and 2).

Similar results for cyanobacteria (cya) have been reported, 11.4 ± 0.5% lipids by Kim and others (2015). *Chlorella vulgaris (Chl)* lipids are reported as 5-12% DWB (Al-Safaar and others, 2016). The lipid fractions extracted from CCA lipid content changed according to the solvent polarity used for extraction (Grima and others, 1999; D'Oca and others, 2011). The methods and appropriate solvents for the disruption of the cell wall are imperative to increase the extraction efficiency.

For all lipid analyses Treatment 1 had less lipids than treatment 2. For Bligh and Dyer method the difference was significant at 95% confidence (p= 0.05), for ASE method there was no significant difference among treatments (p= 0.16), for fat hydrolysis differences were significantly different at 95% confidence (p= 0.007). This is at odds with data that suggests cyanobacteria has low lipid content when compared to Chl. It is possible that the species ratio shifted throughout the 32-day culture growth allowing lipids to accumulate for treatment 2 than treatment 1. In a study on lipid characterization of *Leptolyngbya* sp. ISTCY101 lipid content was reported as 16–21 % dry weight biomass. This study used the growth parameters: BG-11 medium, a semi continuous incubator, cultures were maintained at 30 °C, under continuous fluorescent light (50 μE s^{-1} m^{-2}) (Singh and Thakur, 2014). This study is reported as evidence that cyanobacteria can accumulate high amounts of lipids like their microalgae counterparts. The use future use of a sequential solvent extraction method like that of Andersen and Markham (2006) where extraction was performed first with n-hexane to extract non polar lipids, then ethyl acetate to obtain less polar compounds, and finally methanol was used eventually for more polar compounds (Kokabi, and others 2019) is suggested for future studies. This method could increase lipid yield and provide more clear results of CCA lipids in trt 1 and 2.

Table 6.1. Total Lipid Content by Bligh Dyer Method for CCA

Trt	Avg (g fat/100g algae DWB)
1	0.47 ± 0.10 a
2	0.74 ± 0.10 b

Trt means treatment. Treatment 1 average scalar irradiance of 1041 ± 269.18 μmol m-2 s-1 PAR. Treatment 2 ASI of 430 ± 96.03 μmol m-2 s-1 PAR.

Table 6.2. Total Non-Polar Fat Content by ASE Method for CCA

Trt	Avg (g of fat/ 100 g algae DWB)
1	8.20 ± 1.20 a
2	12.70 ± 2.50 a

Trt means treatment. Treatment 1 average scalar irradiance of 1041 ± 269.18 μmol m-2 s-1 PAR. Treatment 2 ASI of 430 ± 96.03 μmol m-2 s-1 PAR.

Table 6.3. Total Fat Content by Fat Hydrolysis for CCA

Trt	Avg (g of fat/ 100 g algae DWB)
1	7.05 ± 0.51a
2	8.98 ± 0.24 b

Trt means treatment. Treatment 1 average scalar irradiance of 1041 ± 269.18 μmol m-2 s-1 PAR. Treatment 2 ASI of 430 ± 96.03 μmol m-2 s-1 PAR.

6.4.2. Fatty Acid Profile

In each CCA treatment (trt) 10 fatty acids (FA) were identified. Total fat was calculated as the sum of individual fatty acids. C13- C18 Fatty acids were identified, the most abundant being C16:0 Palmitic acid, C18:3 Linolenic acid and, C18:1 Oleic acid. Bai (2012) studied fatty acid composition of CCA and found that Palmitic acid (C16:0) comprised the highest portion of all the FAs, around 30% of total FAs found in CCA. In this study palmitic acid was the most abundant FA found it was 22.55% of the total FAs found in trt 1, and 21.96% of the total FAs found in trt 2. Also, like Bai (2012) FAs smaller than 14 carbons were not detected, except for C13:1 Tridecenoic 1.23% of total FA in CCA. No long-chain ω-3 or ω-6 fatty acids such as eicosapentaenoic acid (EPA) (20:5, n-3) and docosahexaenoic acid (DHA) (22:6, n-3) were

identified in CCA. C18:3 Linolenic acid was the only ω-3 found in both treatments. C16:0 Palmitic acid and C18:0 Stearic acid were the only saturated FAs found.

This coincides with previous studies of *Chlorella Vulgaris* L. and freshwater algae species contained fatty acids C14 – C18 (Liem and Laur, 1977; Harwood and Moore, 1989; Lang and others, 2011; Armenta and others, 2013; Guschina and Harwood, 2016; Matos, 2017; Fernández-Linares and others, 2017). Linoleic acid was found to be present in both *Chlorella* and Cyanobacteria species at relatively high percentages of fatty acids previously (Armenta and others, 2013; Matos, 2017), but was not the dominant fatty acid in CCA.

Benavente-Valdés and others, (2016) and Seyfabadi and others (2011) suggest that saturated fatty acids increase, while monounsaturated and polyunsaturated fatty acids decrease when exposed to increasing irradiance and light duration like that of treatment 1 at 1041 ± 269.18 μmol m^{-2} s^{-1} PAR This study found the opposite effect in CCA fatty acids except for C17:1 Margaroleic acid which is monounsaturated and decreased by 0.11% DWB when light exposure decreased in treatment 2 to 430 ± 96.03 μmol m^{-2} s^{-1} PAR.

Table 6.4. Fatty Acid Profile of CCA

Fatty Acid	Treatment 1 (g FA/ 100g algae)	Trt 1 (% FA in TFA (w/w)	Treatment 2 (g FA/ 100g algae)	Trt 2 (% FA in TFA (w/w)
C13:1 Tridecenoic	0.000	0.00	0.022	1.23
C14:1 Myristoleic	0.026	1.84	0.040	2.23
C16:0 Palmitic	0.318	22.55	0.394	21.96
C16:1 Palmitoleic	0.072	5.11	0.074	4.12
C17:1 Margaroleic	0.064	4.54	0.042	2.34
C18:0 Stearic	0.000	0.00	0.014	0.78
C18:1 Oleic	0.252	17.87	0.348	19.40
C18:2 Linoleic	0.218	15.46	0.126	7.02
C18:3 Linolenic	0.280	19.86	0.358	19.96
C18:4 Octadecatetraenoic	0.030	2.13	0.064	3.57
Other Fatty Acids	0.150	10.64	0.312	17.39
Total (TFA)	1.410	100	1.794	100

Trt means treatment. Treatment 1 average scalar irradiance of 1041 ± 269.18 μmol m-2 s-1 PAR. Treatment 2 ASI of 430 ± 96.03 μmol m-2 s-1 PAR.

6.5. Conclusion

In each CCA treatment (trt) 10 fatty acids (FA) were identified. Total fat was calculated as the sum of individual fatty acids. C13- C18 Fatty acids were identified, the most abundant being C16:0 Palmitic acid, C18:3 Linolenic acid and, C18:1 Oleic acid. Palmitic acid was the most abundant FA found it was 22.55% of the total FAs found in trt 1, and 21.96% of the total FAs found in trt 2. FAs smaller than 14 carbons were not detected, except for C13:1 Tridecenoic was 1.23% of total FA in CCA trt 2. No long-chain ω-3 or ω-6 fatty acids such as eicosapentaenoic acid (EPA) (20:5, n-3) and docosahexaenoic acid (DHA) (22:6, n-3) were identified in CCA. C18:3 Linolenic acid was the only ω-3 found in both treatments. C16:0 Palmitic acid and C18:0 Stearic acid were the only saturated FAs found. Extracted lipid contents were lower than previous studies this could be due to cellular extraction issues. Total lipid content varies greatly depending on polarity of extraction solvent and technique used. C13- C18 Fatty acids were identified, the most abundant being C16:0 Palmitic acid, C18:3 Linolenic acid and, C18:1 Oleic acid. CCA lipids are a viable option for biofuels and creating nutritional and medicinal products due to their ability to accumulate lipids under stress and their plant-like fatty-acid composition.

6.6. References

Al-Safaar, A. T., Al-Rubiaee, G. H., & Salman, S. K. (2016). Effect of pHCondition on the Growth and Lipid Content of Microalgae Chlorella vulgaris&Chroococcus minor. Journal of Scientific & Engineering Research, 7(11), 1139.

Andersen, O. M. and Markham, K. R. 2006. Flavonoids: Chemistry, Biochemistry and Applications. CRC Press Taylor & Francis Group 6000 Broken Sound Parkway NW, Suite 300 Boca Raton, FL.

Araki, S., Eichenberger, W., Sakurai, T., & Sato, N. (1991). Distribution of diacylglycerylhydroxymethyltrimethyl-β-alanine (DGTA) and phosphatidylcholine in brown algae. Plant and cell physiology, 32(5), 623-628.

Bai, R., Gutierrez-Wing, M.T., Negulescu, I.I., Rusch, K.A. (2013) Effect of organic carbon, C:N ratio and light on the growth and lipid productivity of microalgae/cyanobacteria coculture.Engineering in Life Sciences.

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. Canadian journal of biochemistry and physiology, 37(8), 911-917.

Fernández-Linares, L. C., Guerrero Barajas, C., Durán Páramo, E., & Badillo Corona, J. A. (2017). Assessment of *Chlorella vulgaris* and indigenous microalgae biomass with treated wastewater as growth culture medium. Bioresource Technology, 244, 400-406. doi: https://doi.org/10.1016/j.biortech.2017.07.141

Grima, M.E. Medina, R.A., Giménez, G.A. (1999). Recovery of algal PUFAs. Z. Cohen (Ed.), Chemicals from microalgae, Taylor & Francis, London. pp. 108-144.

Guschina, I. A., & Harwood, J. L. (2009). Algal lipids and effect of the environment on their biochemistry. In Lipids in aquatic ecosystems (pp. 1-24). Springer, New York, NY.

Halim, R., Danquah, M. K., & Webley, P. A. (2012). Extraction of oil from microalgae for biodiesel production: A review. Biotechnology Advances, 30(3), 709-732. doi: https://doi.org/10.1016/j.biotechadv.2012.01.001

Harwood, J., & Moore Jr, T. S. (1989). Lipid metabolism in plants. Critical reviews in plant sciences, 8(1), 1-43.

Kiran, B., Pathak, K., Kumar, R., Deshmukh, D., & Rani, N. (2016). Influence of varying nitrogen levels on lipid accumulation in *Chlorella* sp. International journal of environmental science and technology, 13(7), 1823-1832.

Kokabi, M., Yousefzadi, M., Soltani, M., & Arman, M. (2019). Effects of different UV radiation on photoprotective pigments and antioxidant activity of the hot-spring cyanobacterium *Leptolyngbya* cf. fragilis. Phycological Research.

Lang, Imke. Fatty Acid Profiles and Their Distribution Patterns in Microalgae: A Comprehensive Analysis of More than 2000 Strains from the SAG Culture Collection. BMC Plant Biology 11 (2011): 124. PMC.

Nakamura, Y., & Li-Beisson, Y. (Eds.). (2016). Lipids in plant and algae development. Springer International Publishing.

Petkov, G., & Garcia, G. (2007). Which are fatty acids of the green alga *Chlorella*. Biochemical Systematics and Ecology, 35(5), 281-285.

Ramírez-López, C., Chairez, I., & Fernández-Linares, L. (2016). A novel culture medium designed for the simultaneous enhancement of biomass and lipid production by *Chlorella vulgaris* UTEX 26. Bioresource technology, 212, 207-216.

Singh, J., Tripathi, R., & Thakur, I. S. (2014). Characterization of endolithic cyanobacterial strain, Leptolyngbya sp. ISTCY101, for prospective recycling of CO2 and biodiesel production. Bioresource Technology, 166, 345-352. doi:https://doi.org/10.1016/j.biortech.2014.05.055

Van Wychen, S., & Laurens, L. M. L. (2013). Determination of total lipids as fatty acid methyl esters (FAME) by in situ transesterification. Contract, 303, 375-300.

Yao, L., Gerde, J. A., Lee, S.-L., Wang, T., & Harrata, K. A. (2015). Microalgae Lipid Characterization. *Journal of Agricultural and Food Chemistry, 63*(6), 1773-1787. doi:10.1021/jf5050603

Zhu, L. D., Li, Z. H., & Hiltunen, E. (2016). Strategies for Lipid Production Improvement in Microalgae as a Biodiesel Feedstock. BioMed Research International, 2016, 8. doi:10.1155/2016/8792548

Chapter 7. Summary and Conclusion

A thorough review of bioactive compounds present in the species present in Louisiana Native Co-Culture of Microalgae (*Chlorella Vulgaris L.* and Cyanobacteria *Leptolyngbya* sp.) (CCA) was reported. CCA is a viable polyculture for further investigation as a source of food components. This research explored growth parameters that include irradiance, flow cytometry, optical density, pH monitoring, temperature monitoring, growth medium, aeration, and chlorination. Treatment 1 was CCA grown in cultures exposed to average scalar irradiance (ASI) of 1041 ± 269.18 μmol m^{-2} s^{-1} PAR and treatment 2 was CCA grown in cultures exposed to ASI of 430 ± 96.03 μmol m^{-2} s^{-1} PAR. The treatments (irradiance exposure) had the desired response on the CCA species ratio as Trt 1 yielded an average culture ratio of 97.47 ± 1.29% *Chlorella*, and 2.84 ± 1.27% Cyanobacteria. Trt 2 yielded an average culture ratio of 89.85 ± 1.17 *Chlorella*, and 10.64 ± 1.97 Cyanobacteria.

Table 7.1. Summary of CCA Macronutrients Characterized

Macronutrient (g/ 100g algae DWB)	Trt 1	Trt 2
Protein	29.46 ± 6.11	39.67 ± 5.15
Carbohydrates	25.44 ± 6.90	19.28 ± 2.84
Lipids	8.20 ± 1.20	12.70 ± 2.50
Other	36.90*	28.35*

*Estimation of uncharacterized CCA components. Trt means treatment. Treatment 1 average scalar irradiance of 1041 ± 269.18 μmol m-2 s-1 PAR. Treatment 2 ASI of 430 ± 96.03 μmol m-2 s-1 PAR.

Proteins are the largest macronutrient in CCA. The Lowry Assay identified Treatment 1 contained 29.46 ± 6.11 g protein per 100 g of algae DWB and Treatment 2 contained 39.67 ± 5.15 g protein per 100 g of algae DWB; Treatment 2 contained significantly more protein at 95% confidence. Seventeen out of 21 amino acids (AA) were detected in both treatments of CCA. CCA is a complete protein, containing all the essential AA there was no significant difference in amino acid content between treatments at 95% confidence. The molecular mass of extracted

proteins was determined under denaturing conditions by SDS–PAGE bands were identified at 100-110, 90, 52, 33-32, 25, 15, and 13 kDa from these and RUBISCO and phycobiliprotein subunits were hypothesized to be present. MALDI-TOF-MS identified several proteins in CCA with various cellular function such as enzymes, molecular chaperoning, heat shock, protein coding and transcriptional regulators.

Carbohydrates are the second largest macronutrient in CCA. Total sugar content was calculated as 25.44±6.90g/ 100 of algae DWB for treatment 1 (71% of Treatment 1 CCA's carbohydrates are starch, comprised of 23% resistant starch, and 48% non-resistant starch). Total sugar content for treatment 2 was 19.28±2.84 g/ 100 of algae (82% of treatment 2's carbohydrates are starch, comprised of 26% resistant starch, and 56% non-resistant starch). Amylose/amylopectin results conflicted with previous trends that *Chlorella* species synthesize polysaccharides that are mainly hypothesized as amylopectin. Amylose content was 71.62 ± 7.18% w/w and amylopectin content were 28.28 ± 7.18% w/w for treatment 1. Amylose content was 65.85 ± 3.87 % w/w and amylopectin content were 34.15 ± 3.87 % w/w for treatment 2, there was no significant difference among treatments at 95% confidence (p=0.09). Seven monosaccharides were identified and quantified from CCA, the greatest of which were mannose, glucose and galactose. Total monosaccharide content for treatment 1 was identified as 1.36 ± 0.11 g monosaccharide per 100 g of algae DWB, and treatment 2 was 1.44 ± 0.09 g monosaccharide per 100 g of algae DWB. DSC measured the thermal characteristics and enthalpy in CCA extracted starch, it was found to have an increased thermodynamic range when compared to corn starch as it peaks at around 120ºC, indicating the presence of resistant starch. There was no difference between treatments at 95% confidence for any carbohydrate method, this indicates the treatments (irradiance exposure at ASI 1041 ± 269.18 μmol m-2 s-1 PAR [Trt

1] and ASI of 430 ± 96.03 μmol m-2 s-1 PAR [Trt 2]) did not significantly change carbohydrate growth, expression in CCA. The ASI range was relatively high at 1310.18-333.97 μmol m-2 s-1 PAR, this could be why no difference in carbohydrates was observed. It is known that in less light (ASI 80 μmol m-2 s-1 PAR) *Chlorella* growth decrease and cyanobacteria continue to grow and store energy in carbohydrates.

Extracted lipid contents were lower than previous studies this could be due to cellular extraction issues. It was found that total lipid content varies greatly depending on polarity of extraction solvent and technique used. Total lipid content by Bligh Dyer extraction yield the lowest lipid contents (0.47 and 0.74 g fat/ 100 g algae DWB for treatment 1 and 2 respectively), while hexane extraction with Accelerated Solid Extraction (ASE) had the highest total lipid yield for CCA (8.20 ± 1.20 and 12.70 ± 2.50% DWB for treatment 1 and 2).

Fatty acids with 13-18 carbons were identified, the most abundant being C16:0 Palmitic acid, C18:3 Linolenic acid and, C18:1 Oleic acid. In each CCA treatment (trt) 10 fatty acids (FA) were identified. Total fat was calculated as the sum of individual fatty acids. Palmitic acid was the most abundant FA found it was 22.55% of the total FAs found in trt 1, and 21.96% of the total FAs found in trt 2. FAs smaller than 14 carbons were not detected, except for C13:1 Tridecenoic was 1.23% of total FA in CCA trt 2. No long-chain ω-3 or ω-6 fatty acids such as eicosapentaenoic acid (EPA) (20:5, n-3) and docosahexaenoic acid (DHA) (22:6, n-3) were identified in CCA. C18:3 Linolenic acid was the only ω-3 found in both treatments. C16:0 Palmitic acid and C18:0 Stearic acid were the only saturated FAs found. CCA lipids are a viable option for biofuels and creating nutritional and medicinal products due to their ability to accumulate lipids under stress and their plant-like fatty-acid composition.

In conclusion CCA's ability to grow in several irradiance regimes and create substantial biomass while still accumulating valuable macronutrients make it a promising source of bioactive compounds that can be applied in food, feed, nutraceutical, and pharmacological industries. CCA shows promising amounts of amylose that can be applied in food systems as dietary fiber (resistant to digestion).

References

Aakanksha, S.S., Guruprasad, S., Ramachandra T.V., (2010). Diversity of Lipids in Algae. Paper presented at the Lake 2010: Wetlands, Biodiversity and Climate Change, Bangalore, India. Conference Abstract retrieved from a

Abo-Shady, A. M., Mohamed, Y. A., & Lasheen, T. (1993). Chemical composition of the cell wall in some green algae species. Biologia Plantarum, 35(4), 629-632.

Aiken, G. R., D. M. Mcknight, K. A. Thorn, And E. M. Thurman. (1992). Isolation of Hydrophilic Organic Acids from Water Using Nonionic Macroporous Resins. Org. Geochem. 18: 567-573.

Åkerlund, H. E., Jansson, C., & Andersson, B. (1982). Reconstitution of photosynthetic water splitting in inside-out thylakoid vesicles and identification of a participating polypeptide. Biochimica et Biophysica Acta (BBA)-Bioenergetics, 681(1), 1-10.

Al Abdallah, Q., Nixon, B. T., & Fortwendel, J. R. (2016). The Enzymatic Conversion of Major Algal and Cyanobacterial Carbohydrates to Bioethanol. Frontiers in Energy Research, 4(36). doi:10.3389/fenrg.2016.00036

Algae Biomass Organization. (2019). Algae Industry Products & Services Directory. available at: https://algaebiomass.org/resource-center/industry-resources/algae-industry-services-directory/#bigelow (accessed May 2019).

Al-Hasan, R. H., Ali, A. M., & Radwan, S. S. (1989). Effects of light and dark incubation on the lipid and fatty acid composition of marine cyanobacteria. Microbiology, 135(4), 865-872.

Al-Safaar, A. T., Al-Rubiaee, G. H., & Salman, S. K. (2016). Effect of pHCondition on the Growth and Lipid Content of Microalgae Chlorella vulgaris&Chroococcus minor. Journal of Scientific & Engineering Research, 7(11), 1139.

Anagnostidis, K. and Komárek, J. (1988) Modern approach to the classification system of cyanophytes. 3. Oscillatoriales. Arch Hydrobiol Suppl 80, 327–472.

Andersen, O. M. and Markham, K. R. 2006. Flavonoids: Chemistry, Biochemistry and Applications. CRC Press Taylor & Francis Group 6000 Broken Sound Parkway NW, Suite 300 Boca Raton, FL.

Araki, S., Eichenberger, W., Sakurai, T., & Sato, N. (1991). Distribution of diacylglycerylhydroxymethyltrimethyl-β-alanine (DGTA) and phosphatidylcholine in brown algae. Plant and cell physiology, 32(5), 623-628.

Armenta, R. E., & Valentine, M. C. (2013). Single-cell oils as a source of omega-3 fatty acids: an overview of recent advances. Journal of the American Oil Chemists' Society, 90(2), 167-182.

Arteni, A. A., Ajlani, G., & Boekema, E. J. (2009). Structural organisation of phycobilisomes from Synechocystis sp. strain PCC6803 and their interaction with the membrane. Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1787(4), 272-279.

Bai, R. (2012). Lipid Production from a Louisiana Native *Chlorella vulgaris*/*Leptolyngbya* sp. Co-culture for Biofuel Applications. Chemical Engineering Commons. LSU Doctoral Dissertation

Bai, R., Gutierrez-Wing, M.T., Negulescu, I.I., Rusch, K.A. (2013) Effect of organic carbon, C:N ratio and light on the growth and lipid productivity of microalgae/cyanobacteria coculture.Engineering in Life Sciences.

Ball S, Marianne T, Dirick L, Fresnoy M, Delrue B, Decq AA (1991). Chlamydomonas reinhardtii low-starch mutant is defective for 3-phosphoglycerate activation and orthophosphate inhibition of ADP-glucose pyrophosphorylase. Planta 185: 17–26.

Ball, S.G. and Morell, M.K. (2003) From bacterial glycogen to starch: understanding the biogenesis of the plant starch granule. Annu. Rev. Plant Biol. 54: 207–233.

Barbarino, E., & Lourenço, S. O. (2005). An evaluation of methods for extraction and quantification of protein from marine macro-and microalgae. Journal of Applied Phycology, 17(5), 447-460.

Barker, J. P., Cattolico, R. A., & Gatza, E. (2012). Multiparametric Analysis of Microalgae for Biofuels Using Flow Cytometry. White Paper, BD Biosciences.

Barnett, J. Z., Foy, J., Malone, R., Rusch, K. A., & Gutierrez-Wing, M. T. (2017). Impact of light quality on a native Louisiana *Chlorella vulgaris* L./*Leptolyngbya* sp. co-culture. Engineering in Life Sciences, 17(6), 678-685.

Barsanti, L., Coltelli, P., Evangelista, V., Frassanito, A. M., Passarelli, V., Vesentini, N., & Gualtieri, P. (2008). Oddities and curiosities in the algal world. In Algal toxins: nature, occurrence, effect and detection (pp. 353-391). Springer, Dordrecht.

Becker, E. W. (1994). Microalgae: biotechnology and microbiology (Vol. 10). Cambridge University Press.

Becker, E. W. (2007). Micro-algae as a source of protein. Biotechnology advances, 25(2), 207-210.

Benavente-Valdés, J. R., Aguilar, C., Contreras-Esquivel, J. C., Méndez-Zavala, A., & Montañez, J. (2016). Strategies to enhance the production of photosynthetic pigments and lipids in chlorophycae species. Biotechnology Reports, 10, 117–125. http://doi.org/10.1016/j.btre.2016.04.001

Bennett, A. and Bogorad, L. (1973). Complimentary Chromatic Adaptation in a Filamentous Blue-Green Alga. The Journal of Cell Biology, 58, No. 2, 419.

Benson, A. A., R. Wiser, R. A. Ferrari & J. A. Miller, 1958. Photosynthesis of galactolipids. Journal of the American Chemical Society 80: 4740.

Benson, B. C., & Rusch, K. A. (2006). Investigation of the light dynamics and their impact on algal growth rate in a hydraulically integrated serial turbidostat algal reactor (HISTAR). Aquacultural engineering, 35(2), 122-134.

Bernstein, A. M., Ding, E. L., Willett, W. C., & Rimm, E. B. (2012). A meta-analysis shows that docosahexaenoic acid from algal oil reduces serum triglycerides and increases HDL-cholesterol and LDL-cholesterol in persons without coronary heart disease. The Journal of nutrition, 142(1), 99-104.

Bewicke, D., & Potter, B. A. (2009). *Chlorella*: The Emerald Food. Ronin Publishing.

Bhambere, D., A. Gaidhani, K., Harwalkar, M., & S. Nirgude, P. (2015). Lyophilization / Freeze Drying – A Review (Vol. 4).

Birt D., Boylston, T., Hendrich, S., Jane, J., Hollis, J., Li, L., McClelland, J., Moore, S., Phillips, G., Rowling, M., Schalinske, K., Scott, M., Whitley, E. (2013). Resistant Starch: Promise for Improving Human Health. Advances in Nutrition, 4, 587-601.

Bleicher P, Mackin W (1995) Betafectin PGG-glucan: a novel carbohydrate immunomodulator with anti-infective properties. J Biotechnol Healthc 2:207–222

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. Canadian journal of biochemistry and physiology, 37(8), 911-917.

Bold, H.C., 1949. The Morphology of Chlamydomonas Chlamydogama, sp. Nov. Bulletin of the Torrey Botanical Club, 76(2), pp.101-108.

Bonkovsky, H. L., Guo, J. T., Hou, W., Li, T., Narang, T., & Thapar, M. (2013). Porphyrin and heme metabolism and the porphyrias. Comprehensive Physiology.

Borowitzka, M. A. (1999). Commercial production of microalgae: ponds, tanks, tubes and fermenters.

Borowitzka, M. A. (2013). High-value products from microalgae—their development and commercialisation. Journal of Applied Phycology, 25(3), 743-756.

Bouterfas R, Belkoura M, Dauta A (2006). The effects of irradiance and photoperiod on the growth rates of three freshwater green algae isolated from a eutrophic lake. Limnetica 25:647–656

Brennan, L., & Owende, P. (2010). Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. Renewable and sustainable energy reviews, 14(2), 557-577.

Bricker, T. M., & Frankel, L. K. (2002). The structure and function of CP47 and CP43 in photosystem II. Photosynthesis research, 72(2), 131.

Bricker, T. M., Morvant, J., Masri, N., Sutton, H. M., & Frankel, L. K. (1998). Isolation of a highly active photosystem II preparation from Synechocystis 6803 using a histidine-tagged mutant of CP 47. Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1409(1), 50-57.

Bricker, T. M., Odom, W. R., & Queirolo, C. B. (1988). Close association of the 33 kDa extrinsic protein with the apoprotein of CPa1 in photosystem II. FEBS letters, 231(1), 111-117.

Brooks, G., Franklin, S., Avila, J., Decker, S. M., Baliu, E., Rakitsky, W., ... & Norris, L. M. (2010). U.S. Patent Application No. 12/684,893.

Brown, R.M., Jr., & Bold, H.C. (1964). Comparative studies of the algal genera Tetracystis and Chlorococcum. University of Texas Publication No. 6417: 1-213.

Burnap, R. L., Shen, J. R., Jursinic, P. A., Inoue, Y., & Sherman, L. A. (1992). Oxygen yield and thermoluminescence characteristics of a cyanobacterium lacking the manganese-stabilizing protein of photosystem II. Biochemistry, 31(32), 7404-7410.

Capelli, B., & Cysewski, G. R. (2010). Potential health benefits of spirulina microalgae. Nutrafoods, 9(2), 19-26.

Caprioli, R. M., Farmer, T. B., & Gile, J. (1997). Molecular imaging of biological samples: localization of peptides and proteins using MALDI-TOF MS. Analytical chemistry, 69(23), 4751-4760.

Cerón, M.C., Campos, I., Sánchez, J.F., Acién, F.G., Molina, E., Fernández-Sevilla, J.M., 2008. Recovery of lutein from microalgae biomass: development of a process for Scenedesmus almeriensis biomass. J. Agric. Food Chem. 56, 11761–11766.

Chacón-Lee TL, González-Mariño GE (2010) Microalgae for “healthy” foods – possibilities and challenges. Compr Rev Food Sci Food 9(6):655–675

Chakdar, H., & Pabbi, S. (2017). Algal pigments for human health and cosmeceuticals. In Algal Green Chemistry (pp. 171-188). Elsevier.

Chang, Chuan. (2012). Carbohydrates- Comprehensive Studies on Glycobiology and Glycotechnology. InTech. 290-316.

Chen, X., Wu, M., Yang, Q., & Wang, S. (2017). Preparation, characterization of food grade phycobiliproteins from Porphyra haitanensis and the application in liposome-meat system. LWT, 77, 468-474. doi: https://doi.org/10.1016/j.lwt.2016.12.005

Cogdell, R. J. (1978). Carotenoids in photosynthesis. Philosophical Transactions of the Royal Society of London. B, Biological Sciences, 284(1002), 569-579. doi:10.1098/rstb.1978.0090

Corcoran AA, Boeing WJ. (2012). Biodiversity increases the productivity and stability of phytoplankton communities. PloS ONE. 7, e49397. pmid:23173059

Cuellar-Bermudez, S. P., Aguilar-Hernandez, I., Cardenas-Chavez, D. L., Ornelas-Soto, N., Romero-Ogawa, M. A., & Parra-Saldivar, R. (2015). Extraction and purification of high-value metabolites from microalgae: essential lipids, astaxanthin and phycobiliproteins. Microbial biotechnology, 8(2), 190-209.

Cuellar-Bermúdez, S. P., Barba-Davila, B., Serna-Saldivar, S. O., Parra-Saldivar, R., Rodriguez-Rodriguez, J., Morales-Davila, S., . . . Chuck-Hernández, C. (2017). Deodorization of Arthrospira platensis biomass for further scale-up food applications. Journal of the Science of Food and Agriculture, 97(15), 5123-5130. doi:10.1002/jsfa.8391

Cunningham, A. (1993). Analysis of microalgae and cyanobacteria by flow cytometry. In Flow Cytometry in Microbiology (pp. 131-142). Springer, London.

Cyanobacteria. (2017). Funk & Wagnalls New World Encyclopedia, 1p. 1.

Dahiya A, Todd J, McInnis A. Wastewater treatment integrated with algae production for biofuel. In: Gordon R, Seckbach J, editors. The science of algal fuels: cellular origin, life in extreme habitats and astrobiology. Dordrecht: Springer Netherlands; 2012. pp. 447–466.

Davey, H. M., & Kell, D. B. (1996). Flow cytometry and cell sorting of heterogeneous microbial populations: the importance of single-cell analyses. Microbiological reviews, 60(4), 641-696.

de Jesus Raposo, M. F., de Morais, R. M. S. C., & de Morais, A. M. M. B. (2013). Health applications of bioactive compounds from marine microalgae. Life Sciences, 93(15), 479-486. doi: https://doi.org/10.1016/j.lfs.2013.08.002

Delrue B, Fontaine T, Routier F, Decq A, Wieruszeski J-M, Van den Koornhuyse N, Maddelein ML, Fournet B, Ball S (1992) Waxy Chlamydomonas reinhardtii: monocellular algal mutants defective in amylose biosynthesis and granule-bound starch synthase activity accumulate a structurally modified amylopectin. J Bacteriol 174: 3612–3620.

deMan, J. M., & deMan, J. M. (1999). Color. Principles of Food Chemistry, 229-262.

Dhillon, M. K., Kumar, S., & Gujar, G. T. (2014). A common HPLC-PDA method for amino acid analysis in insects and plants. http://nopr.niscair.res.in/handle/123456789/25161

Domozych, D., Ciancia, M., Fangel, J. U., Mikkelsen, M. D., Ulvskov, P., & Willats, W. G. (2012). The cell walls of green algae: a journey through evolution and diversity. Frontiers in plant science, 3, 82.

Ebeling, M. E. (1968). The Dumas method for nitrogen in feeds. Journal of the Association of Official Analytical Chemists, 51, 766-770.

Eonseon, J., Polle, J. E. W., & Lee, H. K. S., M. Hyun, and M. Chang. 2003. Xanthophylls in microalgae: from biosynthesis to biotechnological mass production and application. J. Microbiol. Biotechnol, 13(2), 165-174.

Fassett R, Coombes J (2011) Astaxanthin: a potential therapeutic agent in cardiovascular disease. Mar Drugs 9(3):447–465

Fernández-Linares, L. C., Guerrero Barajas, C., Durán Páramo, E., & Badillo Corona, J. A. (2017). Assessment of *Chlorella vulgaris* and indigenous microalgae biomass with treated wastewater as growth culture medium. Bioresource Technology, 244, 400-406. doi: https://doi.org/10.1016/j.biortech.2017.07.141

Ferrell, J., & Sarisky-Reed, V. (2010). National algal biofuels technology roadmap (No. DOE/EE-0332). EERE Publication and Product Library.

Flaibani A, Olsen Y, Painter TJ (1989) Polysaccharides in desert reclamation: compositions of exocellular proteoglycan complexes produced by filamentous blue-green and unicellular green edaphic algae. Carbohydr Res 190(2):235–248

Fleurence, J. (1999). Seaweed proteins: biochemical, nutritional aspects and potential uses. Trends in food science & technology, 10(1), 25-28.

Food and Agricultural Organization/World Health Organization. (1973). Energy and protein requirement: Report of a Joint FAO/WHO ad hoc Expert Committee, vol. 52, FAO.

Fowden, L. (1952). The composition of the bulk proteins of *Chlorella*. Biochemical Journal, 50(3), 355.

Galasso, C., Corinaldesi, C., & Sansone, C. (2017). Carotenoids from Marine Organisms: Biological Functions and Industrial Applications. Antioxidants, 6(4), 96. doi:10.3390/antiox6040096

Gallant, D.J., Bouchet, B. and Baldwin, P.M. (1997) Microscopy of starch: evidence of a new level of granule organization. Carbohydr. Polym. 32: 177–191.

Geresh, S., Dubinsky, O., Arad, S., Christiaen, D., & Glaser, R. (1990). Structure of 3-O- (α-d-glucopyranosyluronic acid)-l-galactopyranose, an aldobiouronic acid isolated from the polysaccharides of various unicellular red algae. Carbohydrate Research, 208, 301-305. doi: https://doi.org/10.1016/0008-6215(90)80116-K

Gidley, M.J. and Bulpin, P.V. (1987) Crystallisation of malto-oligosaccharides as models of the crystalline forms of starch: minimum chain-length requirement for the formation of double helices. Carbohydr. Res. 161: 291–300.

Gifuni, I., Olivieri, G., Krauss, I. R., D'Errico, G., Pollio, A., & Marzocchella, A. (2017). Microalgae as new sources of starch: isolation and characterization of microalgal starch granules. Chemical Engineering Transactions, 57, 1423-1428.

Gill, P., Tohidu Moghadam, T., Ranjbar, B. (2010). Differential Scanning Calorimetry Techniques: Applications in Biology and Nanoscience. Biomolecular Techniques. 21, 167-193.

Glazer, A. N. (1994). Phycobiliproteins — a family of valuable, widely used fluorophores. Journal of Applied Phycology, 6(2), 105-112. doi:10.1007/BF02186064

Glazer, A.N., 1989. Light guides. Directional energy transfer in a photosynthetic antenna. J. Biol. Chem. 264:1-4

Goo, B. G., Baek, G., Choi, D. J., Park, Y. I., Synytsya, A., Bleha, R., ... & Park, J. K. (2013). Characterization of a renewable extracellular polysaccharide from defatted microalgae Dunaliella tertiolecta. Bioresource technology, 129, 343-350.

Graham, L. E., and L. W. Wilcox. 2000. *Algae*. Upper Saddle River: Prentice Hall.

Green, R. E., Sosik, H. M., Olson, R. J., & DuRand, M. D. (2003). Flow cytometric determination of size and complex refractive index for marine particles: comparison with independent and bulk estimates. Applied Optics, 42(3), 526-541.

Grima, M.E. Medina, R.A., Giménez, G.A. (1999). Recovery of algal PUFAs. Z. Cohen (Ed.), Chemicals from microalgae, Taylor & Francis, London. pp. 108-144.

Guckert, J. B., & Cooksey, K. E. (1990). Triglyceride accumulation and fatty acid profile changes in *Chlorella* (Chlorophyta) during high Ph-induced cell cycle Inhibition 1. Journal of Phycology, 26(1), 72-79.

Guedes, A.C., Sousa-Pinto, I., Malcata, F.X. (2015). Application of protein of microalgae to aquafeed in Handbook of Marine Microalgae Biotechnology Advances. Se-Kwan Kim (ed.). Chp. 8, pp. 93-126.

Guschina, I. A., & Harwood, J. L. (2006). Lipids and lipid metabolism in eukaryotic algae. Progress in Lipid Research, 45(2), 160-186. doi:https://doi.org/10.1016/j.plipres.2006.01.001

Guschina, I. A., & Harwood, J. L. (2009). Algal lipids and effect of the environment on their biochemistry. In Lipids in aquatic ecosystems (pp. 1-24). Springer, New York, NY.

Gutierrez-Wing, M.T, (unpublished work). Notes from meetings. Verbal information provided based on experience. Department of Civil and Environmental Engineering. Louisiana State University.

Guzman, S., Gato, A., Lamela, M., Freire-Garabal, M., & Calleja, J. M. (2003). Anti-inflammatory and immunomodulatory activities of polysaccharide from *Chlorella* stigmatophora and Phaeodactylum tricornutum. Phytotherapy Research, 17(6), 665-670.

Habib, M. A. B. (2008). Review on culture, production and use of Spirulina as food for humans and feeds for domestic animals and fish. FAO Fisheries and Aquaculture Circular. No. 1034. http://www.fao.org/3/i0424e/i0424e00.pdf.

Halim, R., Danquah, M. K., & Webley, P. A. (2012). Extraction of oil from microalgae for biodiesel production: A review. Biotechnology Advances, 30(3), 709-732. doi: https://doi.org/10.1016/j.biotechadv.2012.01.001

Hall, N. G., & Schönfeldt, H. C. (2013). Total nitrogen vs. amino-acid profile as indicator of protein content of beef. Food Chemistry, 140(3), 608-612. doi: https://doi.org/10.1016/j.foodchem.2012.08.046

Harwood J.L. (1998) Membrane Lipids in Algae. In: Paul-André S., Norio M. (eds) Lipids in Photosynthesis: Structure, Function and Genetics. Advances in Photosynthesis and Respiration, vol 6. Springer, Dordrecht

Harwood, J., & Moore Jr, T. S. (1989). Lipid metabolism in plants. Critical reviews in plant sciences, 8(1), 1-43.

Hejazi, M. A., & Wijffels, R. H. (2004). Milking of microalgae. Trends Biotechnol, 22(4), 189-194. doi: 10.1016/j.tibtech.2004.02.009

Henríquez, V., Escobar, C., Galarza, J., & Gimpel, J. (2016). Carotenoids in microalgae Carotenoids in Nature (pp. 219-237): Springer.

Ho, S.-H., Huang, S.-W., Chen, C.-Y., Hasunuma, T., Kondo, A., & Chang, J.-S. (2013). Characterization and optimization of carbohydrate production from an indigenous microalga *Chlorella vulgaris* FSP-E. Bioresource Technology, 135(Supplement C), 157-165. doi: https://doi.org/10.1016/j.biortech.2012.10.100

Hyka, P., Lickova, S., Přibyl, P., Melzoch, K., & Kovar, K. (2013). Flow cytometry for the development of biotechnological processes with microalgae. Biotechnology advances, 31(1), 2-16.

Jeffrey SW, Llewellyn CA, Barlow RG, Mantoura RFC (1997). Pigment processes in the sea: a selected bibliography. In: Jeffrey SW, Mantoura RFC, Wright SW (eds) Phytoplankton pigments in oceanography. SCOR-UNESCO, Paris, p 167–178

Jenkins, P.J., Cameron, R.E. and Donald, A.M. (1993) A universal feature in the structure of starch granules from different botanical sources. Starch 45: 417–420.

Jiang, Yu, "Effects of Amino Acids and Fatty Acids on Rice Starch Properties: Thermal, Pasting, Resistant Starch and Structural Characterization" (2013). LSU Doctoral Dissertations. 1943. https://digitalcommons.lsu.edu/gradschool_dissertations/1943

Juliano, B. (1971). A simplified assay for milled-rice amylose. Cereal Sci. Today, 16, 334-360.

Kainuma, K. and French, D. (1972) Naegeli amylodextrin and its relationships to starch granule structure. II. Role of water in crystallization of B-starch. Biopolymers 11: 2241–2250.

Karkacier, M., Erbas, M., Uslu, M. K., & Aksu, M. (2003). Comparison of different extraction and detection methods for sugars using amino-bonded phase HPLC. Journal of chromatographic science, 41(6), 331-333.

Kent, M., Welladsen, H. M., Mangott, A., & Li, Y. (2015). Nutritional evaluation of Australian microalgae as potential human health supplements. PloS one, 10(2), e0118985.

Khairy, H. M., Ali, E. M., & Dowidar, S. M. (2011). Comparative effects of autotrophic and heterotrophic growth on some vitamins, 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity, amino acids and protein profile of *Chlorella vulgaris* Beijerinck. African Journal of Biotechnology, 10(62), 13514-13519.

Kim, J., Choi, W., Jeon, S. , Kim, T. , Park, A. , Kim, J. , Heo, S. , Oh, C. , Shim, W. and Kang, D. (2015), Isolation and characterization of *Leptolyngbya* sp. KIOST-1, a basophilic and euryhaline filamentous cyanobacterium from an open paddle-wheel raceway Arthrospira culture pond in Korea. J Appl Microbiol, 119: 1597-1612. doi:10.1111/jam.12961

Kiran, B., Pathak, K., Kumar, R., Deshmukh, D., & Rani, N. (2016). Influence of varying nitrogen levels on lipid accumulation in *Chlorella* sp. International journal of environmental science and technology, 13(7), 1823-1832.

Kissoudi, M. Sarakatsianos, I., Samanidou, V. (2018). Isolation and purification of food-grade C-phycocyanin from Arthrospira platensis and its determination in confectionery by HPLC with diode array detection. J Separation Sci. 41(4): 975-981.

Koh, J., Xu, Z. and Wicker, L. (2018), Blueberry Pectin Extraction Methods Influence Physico-Chemical Properties. Journal of Food Science, 83: 2954-2962. doi:10.1111/1750-3841.14380

Kokabi, M., Yousefzadi, M., Soltani, M., & Arman, M. (2019). Effects of different UV radiation on photoprotective pigments and antioxidant activity of the hot-spring cyanobacterium *Leptolyngbya* cf. fragilis. Phycological Research.

Komárek J. (1992): Diversita a moderní klasifikace sinic (Cyanoprocaryota) [Diversity and modern classification of Cyanobacteria (Cyanoprokaryota). - inaugural dissertation, not published

Kuriyan, J., Krishna, T. S. R., Wong, L., Guenther, B., Pahler, A., Williams, C. H., & Model, P. (1991). Convergent evolution of similar function in two structurally divergent enzymes. Nature, 352(6331), 172-174. doi:10.1038/352172a0

Kuwabara, T., & Murata, N. (1979). Purification and characterization of 33 kilodalton protein of spinach chloroplasts. Biochimica et Biophysica Acta (BBA)-Protein Structure, 581(2), 228-236.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, London 227, 680-685.

Lang, Imke. Fatty Acid Profiles and Their Distribution Patterns in Microalgae: A Comprehensive Analysis of More than 2000 Strains from the SAG Culture Collection. BMC Plant Biology 11 (2011): 124. PMC.

Lee, R. F., & Loeblich III, A. R. (1971). Distribution of 21: 6 hydrocarbon and its relationship to 22: 6 fatty acid in algae. Phytochemistry, 10(3), 593-602.

Lee, W. C. Y., Lee, W. H., & Rosenbaum, M. (1998). *Chlorella*: McGraw-Hill Education.

Levy, L. W. (2001). U.S. Patent No. 6,191,293. Washington, DC: U.S. Patent and Trademark Office.

Li, Y., Ghasemi Naghdi, F., Garg, S., Adarme-Vega, T. C., Thurecht, K. J., Ghafor, W. A., … Schenk, P. M. (2014). A comparative study: the impact of different lipid extraction methods on current microalgal lipid research. Microbial Cell Factories, 13, 14. http://doi.org/10.1186/1475-2859-13-14

Li, Y., Horsman, M., Wu, N., Lan, C. Q., & Dubois-Calero, N. (2008). Biofuels from microalgae. Biotechnology progress, 24(4), 815-820.

Liang S., Liu X., Chen F., Chen Z. (2004) Current microalgal health food R & D activities in China. In: Ang P.O. (eds) Asian Pacific Phycology in the 21st Century: Prospects and Challenges. Developments in Hydrobiology, vol 173. Springer, Dordrecht

Liem, P. Q., & Laur, M. H. (1977). Structures, teneurs et compositions des esters sulfuriques, sulfoniques, phosphoriques des glycosyldiglycérides de trois fucacées. Biochimie, 58(11-12), 1367-1380.

López, C. V. G., García, M. D. C. C., Fernández, F. G. A., Bustos, C. S., Chisti, Y., & Sevilla, J. M. F. (2010). Protein measurements of microalgal and cyanobacterial biomass. Bioresource technology, 101(19), 7587-7591.

Lourenço, S. O., Barbarino, E., De-Paula, J. C., Pereira, L. O. D. S., & Marquez, U. M. L. (2002). Amino acid composition, protein content and calculation of nitrogen-to-protein conversion factors for 19 tropical seaweeds. Phycological Research, 50(3), 233-241.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. Journal of biological chemistry, 193(1), 265-275.

Maddocks, S. E., & Oyston, P. C. (2008). Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins. Microbiology, 154(Pt 12), 3609-3623. doi:10.1099/mic.0.2008/022772-0

Malkin, R., & Niyogi, K. (2000). Biochemistry and molecular biology of plants. Rockville, MD, USA: American Society of Plant Physiologists, 568-628.

Manners, D.J. (1991) Recent developments in our understanding of glycogen structure. Carbohydr. Polym. 16: 37–82.

Marie, D., Simon, N., & Vaulot, D. (2005). Phytoplankton cell counting by flow cytometry. Algal culturing techniques, 1, 253-267.

Markou, G., Angelidaki, I., & Georgakakis, D. (2012). Microalgal carbohydrates: an overview of the factors influencing carbohydrates production, and of main bioconversion technologies for production of biofuels. Applied microbiology and biotechnology, 96(3), 631-645.

Matos, Â. P. (2017). The Impact of Microalgae in Food Science and Technology. Journal of the American Oil Chemists' Society, 94(11), 1333-1350.

Mendoza, H., De la Jara, A., Freijanes, K., Carmona, L., Ramos, A. A., de Sousa Duarte, V., ... & Carlos, J. (2008). Characterization of Dunaliella salina strains by flow cytometry: a new approach to select carotenoid hyperproducing strains. Electronic Journal of Biotechnology, 11(4), 5-6.

Moheimani, N.R. J Appl Phycol (2013) 25: 387. https://doi.org/10.1007/s10811-012-9873-6

Mohtashamian, Marjan Sadat, "The Use of a Mixed *Chlorella* Cyanobacteria Culture as a Protein Source for Aquaculture" (2012). LSU Master's Theses. 4288. https://digitalcommons.lsu.edu/gradschool_theses/4288

Monsur, H. A., Jaswir, I., Simsek, S., Amid, A., & Alam, Z. (2017). Chemical structure of sulfated polysaccharides from brown seaweed (Turbinaria turbinata). International Journal of Food Properties, 20(7), 1457-1469. doi:10.1080/10942912.2016.1211144

Morel, A. (1991). Optics of marine particles and marine optics. In Particle analysis in oceanography (pp. 141-188). Springer, Berlin, Heidelberg.

Morris, H. J., Carrillo, O., Almarales, A., Bermúdez, R. C., Lebeque, Y., Fontaine, R., ... & Beltrán, Y. (2007). Immunostimulant activity of an enzymatic protein hydrolysate from green microalga *Chlorella vulgaris* L. on undernourished mice. Enzyme and Microbial Technology, 40(3), 456-460.

Morrison, D. J., & Preston, T. (). Formation of short chain fatty acids by the gut microbiota and their impact on human metabolism. Gut microbes, 7(3), 189–200. doi:10.1080/19490976.2015.1134082

Mossé, J. 1990. Nitrogen to protein conversion factor for ten cereals and six legumes or oilseeds. A reappraisal of its definition and determination. Variation according to species and to seed proteic content. J. Agric. Food Chem. 38: 18–24.

Muller-Feuga, A. (1996). Marine microalgae. The stakes of research. French Research Institute for the Exploitation of the Sea, Plouzané.

Murakami, C., Takahashi, J., Shimpo, K., Maruyama, T., & Niiya, I. (1997). Lipids and fatty acid compositions of *Chlorella*. Journal of Japan Oil Chemists' Society, 46(4), 423-427.

Nair, D., Krishna, J. G., Panikkar, M. V. N., Nair, B. G., Pai, J. G., & Nair, S. S. (2018). Identification, purification, biochemical and mass spectrometric characterization of novel phycobiliproteins from a marine red alga, Centroceras clavulatum. International Journal of Biological Macromolecules, 114, 679-691. doi: https://doi.org/10.1016/j.ijbiomac.2018.03.153

Nakamura, Y. (2002) Towards a better understanding of the metabolic system for amylopectin biosynthesis in plants: rice endosperm as a model tissue. Plant Cell Physiol. 43: 718–725.

Nakamura, Y., & Li-Beisson, Y. (Eds.). (2016). Lipids in plant and algae development. Springer International Publishing.

Nakamura, Y., Takahashi, J.-i., Sakurai, A., Inaba, Y., Suzuki, E., Nihei, S., . . . Kurano, N. (2005). Some Cyanobacteria Synthesize Semi-Amylopectin Type α-Polyglucans Instead of Glycogen. Plant and Cell Physiology, 46(3), 539-545. doi:10.1093/pcp/pci045

Neori A. "Green water" microalgae: the leading sector in world aquaculture. J Appl Phycol. 2011; 23: 143–149.

Nicoletti M. (2016). Microalgae Nutraceuticals. Foods (Basel, Switzerland), 5(3), 54. doi:10.3390/foods5030054

Nielsen S.S., (2010). Food Analysis Laboratory Manual, 2nd ed., Springer US, New York.

Nomoto K, Yokokura T, Satoh H, Mutai M (1983) Anti-tumor effect by oral administration of *Chlorella* extract, PCM-4 by oral admission (article in Japanese). Gan To Kagaku Zasshi 10:781–785

Ogawa, K., Ikeda, Y., & Kondo, S. (1999). A new trisaccharide, α-D-glucopyranuronosyl-(1→3)-α-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranose from *Chlorella vulgaris* (Vol. 321).

Ogawa, M. A., & Parra-Saldivar, R. (2015). Extraction and purification of high-value metabolites from microalgae: essential lipids, astaxanthin and phycobiliproteins. Microbial biotechnology, 8(2), 190-209.

Olson, R. J., Vaulot, D., & Chisholm, S. W. (1985). Marine phytoplankton distributions measured using shipboard flow cytometry. Deep Sea Research Part A. Oceanographic Research Papers, 32(10), 1273-1280.

Ortiz-Tena, J. G., Rühmann, B., Schieder, D., & Sieber, V. (2016). Revealing the diversity of algal monosaccharides: Fast carbohydrate fingerprinting of microalgae using crude biomass and showcasing sugar distribution in *Chlorella vulgaris* by biomass fractionation. Algal Research, 17, 227-235. doi: https://doi.org/10.1016/j.algal.2016.05.008

Park, Y., Je, K.-W., Lee, K., Jung, S.-E., et al., Growth promotion of *Chlorella* ellipsoidea by co-inoculation with Brevundimonas sp. isolated from the microalga. Hydrobiologia 2008, 598, 219-228.

Patel, A., Mishra, S., Pawar, R., & Ghosh, P. K. (2005). Purification and characterization of C-Phycocyanin from cyanobacterial species of marine and freshwater habitat. Protein Expression and Purification, 40(2), 248-255. doi: https://doi.org/10.1016/j.pep.2004.10.028

Petkov, G., & Garcia, G. (2007). Which are fatty acids of the green alga *Chlorella*. Biochemical Systematics and Ecology, 35(5), 281-285.

Pohl, P. and Zurheide, F. (1979b) Control of fatty acid and lipid formation in Baltic marine algae by environmental factors. In Advances in the Biochemistry and Physiology of Plant Lipids (Appelqvist, L.A. and Liljenberg, C., eds). Amsterdam: Elsevier, pp. 427–432.

Price, C. A. (1965). A membrane method for determination of total protein in dilute algal suspensions. Analytical biochemistry, 12(2), 213-218.

Priyadarshani I, Biswajit R (2012) Commercial and industrial applications of micro algae – a review. J Algal Biomass Utln 3(4):89–100

Pulz, O. (2001). Photobioreactors: production systems for phototrophic microorganisms. Applied microbiology and biotechnology, 57(3), 287-293.

Pulz, O., & Gross, W. (2004). Valuable products from biotechnology of microalgae. Applied microbiology and biotechnology, 65(6), 635-648.

Ral, J.-P., Derelle, E., Ferraz, C., Wattebled, F., Farinas, B., Corellou, F., . . . Ball, S. (2004). Starch Division and Partitioning. A Mechanism for Granule Propagation and Maintenance in the Picophytoplanktonic Green Alga <em>Ostreococcus tauri</em&gt. Plant Physiology, 136(2), 3333. doi:10.1104/pp.104.044131

Ramírez-López, C., Chairez, I., & Fernández-Linares, L. (2016). A novel culture medium designed for the simultaneous enhancement of biomass and lipid production by *Chlorella vulgaris* UTEX 26. Bioresource technology, 212, 207-216.

Rasmussen, U., Nilsson, M., 2003. Cyanobacterial Diversity and Specificity in Plant Symbioses.Cyanobacteria in Symbiosis, in: Rai, A.N., Bergman, B., Rasmussen, U. (Eds.). Springer Netherlands, pp. 313-328.

Raven J.A., Beardall J. (2003) Carbohydrate Metabolism and Respiration in Algae. In: Larkum A.W.D., Douglas S.E., Raven J.A. (eds) Photosynthesis in Algae. Advances in Photosynthesis and Respiration, vol 14. Springer, Dordrecht

Rioux, L. E., Turgeon, S. L., & Beaulieu, M. (2007). Characterization of polysaccharides extracted from brown seaweeds. Carbohydrate polymers, 69(3), 530-537.

Rismani-Yazdi, H., Haznedaroglu, B. Z., Bibby, K., & Peccia, J. (2011). Transcriptome sequencing and annotation of the microalgae Dunaliella tertiolecta: pathway description and gene discovery for production of next-generation biofuels. BMC genomics, 12(1), 148.

Román, R. B., Alvarez-Pez, J. M., Fernández, F. A., & Grima, E. M. (2002). Recovery of pure B-phycoerythrin from the microalga Porphyridium cruentum. Journal of Biotechnology, 93(1), 73-85.

Rowan, K. S. (1989). Photosynthetic pigments of algae: CUP Archive.

Roy, H., & Cannon, S. (1988). Ribulose bisphosphate carboxylase assembly: what is the role of the large subunit binding protein. Trends in biochemical sciences, 13(5), 163-165.

Rypniewski, W. R., Breiter, D. R., Benning, M. M., Wesenberg, G., Oh, B. H., Markley, J. L., . . . Holden, H. M. (1991). Crystallization and structure determination of 2.5-.ANG. resolution of the oxidized iron-sulfur [2Fe-2S] ferredoxin isolated from Anabaena 7120. Biochemistry, 30(17), 4126-4131. doi:10.1021/bi00231a003

Sabine, C. L., & Feely, R. A. (2007). 3 The Oceanic Sink for Carbon Dioxide. Greenhouse Gas Sinks, 31.

Safi, C., Zebib, B., Merah, O., Pontalier, P.-Y., & Vaca-Garcia, C. (2014). Morphology, composition, production, processing and applications of *Chlorella vulgaris*: A review. Renewable and Sustainable Energy Reviews, 35, 265-278. doi: https://doi.org/10.1016/j.rser.2014.04.007

Schägger, H., & Von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Analytical biochemistry, 166(2), 368-379.

Seckbach, J. (1999). Cellular origin, life in extreme habitats and astrobiology.

Sensen, C. W., Heimann, K., & Melkonian, M. (1993). The production of clonal and axenic cultures of microalgae using fluorescence-activated cell sorting. European Journal of Phycology, 28(2), 93-97.

Seo, Y. C., Choi, W. S., Park, J. H., Park, J. O., Jung, K.-H., & Lee, H. Y. (2013). Stable Isolation of Phycocyanin from Spirulina platensis Associated with High-Pressure Extraction Process. International Journal of Molecular Sciences, 14(1), 1778-1787.

Seyfabadi, Z. R., Zahra Amini Khoeyi. (2011). Protein, fatty acid, and pigment content of *Chlorella vulgaris* L. under different light regimes. Journal of Applied Phycology, 23(4), 721-726. doi:10.1007/s10811-010-9569-8

Sharma, N. K., Rai, A. K., & Stal, L. J. (2013). Cyanobacteria: an economic perspective. John Wiley & Sons.

Shepherd R, Rockey J, Sutherland IW, Roller S (1995) Novel bioemulsifiers from microorganisms for use in foods. J Biotechnol 40(3):207–217

Shi, Y. J. Yang, S.F. Hu, Q. (2007). Purification and identification of polysaccharide derived from *Chlorella* pyrenoidosa. Food Chemistry, 103 (2007) 101–105. doi:10.1016/j.foodchem.2006.07.028

Shimura, Y., Hirose, Y., Misawa, N., Osana, Y., Katoh, H., Yamaguchi, H., & Kawachi, M. (2015). Comparison of the terrestrial cyanobacterium *Leptolyngbya* sp. NIES-2104 and the freshwater *Leptolyngbya* boryana PCC 6306 genomes. DNA Research, 22(6), 403-412.

Sichina, W. J. (2000). Use of DSC for the Characterization of Starches. PerkinElmer Instruments.

Silaban, A. G. (2012). Growth rate and productivity of a Louisiana native microalgae/cyanobacteria co-culture: Feasibility for use in industrial biotechnology applications. Louisiana State University Graduate School Digital Commons. Dept of Civil and Environmental Engineering. Master's Thesis.

Silaban, A. G. (2013). Growth rate and productivity of a Louisiana native microalgae/cyanobacteria co-culture: Feasibility for use in industrial biotechnology applications. LSU Master's Thesis Commons. pp. 202.

Singh, J., Tripathi, R., & Thakur, I. S. (2014). Characterization of endolithic cyanobacterial strain, Leptolyngbya sp. ISTCY101, for prospective recycling of CO2 and biodiesel production. Bioresource Technology, 166, 345-352. doi:https://doi.org/10.1016/j.biortech.2014.05.055

Slocombe, S. P., Ross, M., Thomas, N., McNeill, S., & Stanley, M. S. (2013). A rapid and general method for measurement of protein in micro-algal biomass. Bioresource technology, 129, 51-57.

Spolaore, P., Joannis-Cassan, C., Joannis-Cassan, E., Isambert, A., Commercial applications of microalgae – review. J. Biosci. Bioeng. 2006, 101, 87–96.

Starr, R. C., & Zeikus, J. A. (1993). Utex—The Culture Collection of Algae at The University of Texas At Austin 1993 List of Cultures 1. Journal of Phycology, 29, 1-106.

Stock, A. M., Robinson, V. L., & Goudreau, P. N. (2000). Two-Component Signal Transduction. Annual Review of Biochemistry, 69(1), 183-215. doi: 10.1146/annurev.biochem.69.1.183

Sui, Z., Gizaw, Y., & BeMiller, J. N. (2012). Extraction of polysaccharides from a species of *Chlorella*. Carbohydrate Polymers, 90(1), 1-7. doi: http://dx.doi.org/10.1016/j.carbpol.2012.03.062

Swanson, R. V., & Glazer, A. N. (1990). Separation of phycobiliprotein subunits by reverse-phase high-pressure liquid chromatography. Analytical Biochemistry, 188(2), 295-299. doi: https://doi.org/10.1016/0003-2697(90)90609-D

Tang, X. S., & Satoh, K. (1985). The oxygen-evolving photosystem II core complex. FEBS letters, 179(1), 60-64.

Tate, John Joseph, "Differential Gene Expression in a Louisiana Strain of Microalgae" (2012). LSU Master's Theses. 3945. https://digitalcommons.lsu.edu/gradschool_theses/3945

Tate, J. J., Gutierrez-Wing, M. T., Rusch, K. A., & Benton, M. G. (2013). Gene expression analysis of a Louisiana native *Chlorella vulgaris* L. (Chlorophyta)/*Leptolyngbya* sp. (Cyanobacteria) co-culture using suppression subtractive hybridization. Engineering in Life Sciences, 13(2), 185-193. doi:10.1002/elsc.201200063

Thompson, D.B. (2000) On the non-random nature of amylopectin branching. Carbohydr. Polym. 43: 223–239

Thrive Algae. (2017). Thrive Algae Oil. Visited 4/16/17. http://www.thrivealgae.com/resources/

Trabelsi L, M'sakni NH, Ben Ouada H, Bacha H, Roudesli S. 2009. Partial characterization of extracellular polysaccharides from the CyanobacteriumArthrospira platensis. Biotech Biop Eng.;14:27–31.

Trask, B. J., Van den Engh, G. J., & Elgershuizen, J. H. B. W. (1982). Analysis of phytoplankton by flow cytometry. Cytometry: The Journal of the International Society for Analytical Cytology, 2(4), 258-264.

Ursu, A. V., Marcati, A., Sayd, T., Sante-Lhoutellier, V., Djelveh, G., & Michaud, P. (2014). Extraction, fractionation and functional properties of proteins from the microalgae *Chlorella vulgaris*. Bioresource technology, 157, 134-139.

UTEX Culture Collection of Algae. (2016). Bold 1NV Medium. Algal Media Recipes. https://utex.org/products/bold-1nv-medium.

Van Wychen, S., & Laurens, L. M. L. (2013). Determination of total lipids as fatty acid methyl esters (FAME) by in situ transesterification. Contract, 303, 375-300.

Vertes, A.A., Inui, M., Yukawa, H., 2008. Technological options for biological fuel ethanol. J. Mol. Microbiol. Biotechnol. 15, 16–30.

Wada, H., & Murata, N. (1998). Membrane lipids in cyanobacteria. In Lipids in photosynthesis: structure, function and genetics (pp. 65-81). Springer, Dordrecht.

Waggoner, B., & Speer, B. R. (1999). Photosynthetic Pigments. Website: http://www. ucmp. berkeley. edu/glossary/gloss3/pigments. html.

Wang, X., Liu, X.H., Wang, G.Y., 2011. Two-stage hydrolysis of invasive algal feedstock for ethanol fermentation. J. Integr. Plant. Biol. 53 (3),246–252.

Wydrzynski, T. J., & Satoh, K. (2005). Photosystem II: The light-driven water: plastoquinone oxidoreductase. Dordrecht: Springer.

Xu, H., Miao, X., Wu, Q., High quality biodiesel production from a microalga *Chlorella* protothecoides by heterotrphic growth in fermenters. (2006). J. Biotech. 126, 499–507

Yao, L., Gerde, J. A., Lee, S.-L., Wang, T., & Harrata, K. A. (2015). Microalgae Lipid Characterization. *Journal of Agricultural and Food Chemistry, 63*(6), 1773-1787. doi:10.1021/jf5050603

Yentsch, C. M., Horan, P. K., Muirhead, K., Dortch, Q., Haugen, E., Legendre, L., ... & Spinrad, R. W. (1983). Flow cytometry and cell sorting: A technique for analysis and sorting of aquatic particles1. Limnology and Oceanography, 28(6), 1275-1280.

Yeoh, H. H. and Truong, V. D. 1996. Protein contents, amino acid compositions and nitrogen-to-protein conversion factors for cassava roots. J. Sci. Food Agric. 70: 51–4.

Yim, J. H., Kim, S. J., Ahn, S. H., & Lee, H. K. (2003). Optimal conditions for the production of sulfated polysaccharide by marine microalga Gyrodinium impudicum strain KG03. Biomolecular engineering, 20(4-6), 273-280.

Yu, M. H. and Glazer, N. A., Cyanobacterial Phycobilisomes. Role of the Linker Polypeptides in the Assembly of Phycocyanin. (1982). The Journal of Biological Chemistry, 257, No. 7, 3429.

Zeeman S, Northrop F, Smith AM, ap Rees T (1998) A starch-accumulating mutant of Arabidopsis thaliana deficient in a chloroplastic starch hydrolyzing enzyme. Plant J 15: 357–365.

Zhang, X. (2015). Microalgae removal of CO2 from flue gas. Clean coal technology research reports, April. Retrieved from http://bookshop. iea-coal. org. uk/reports/ccc-250/83697, 244.

Zhu, L. D., Li, Z. H., & Hiltunen, E. (2016). Strategies for Lipid Production Improvement in Microalgae as a Biodiesel Feedstock. BioMed Research International, 2016, 8. doi:10.1155/2016/8792548

Appendix. Supplemental MALDI-TOF-MS Data

A.1. MALDI-TOF-MS Spectra and Tables

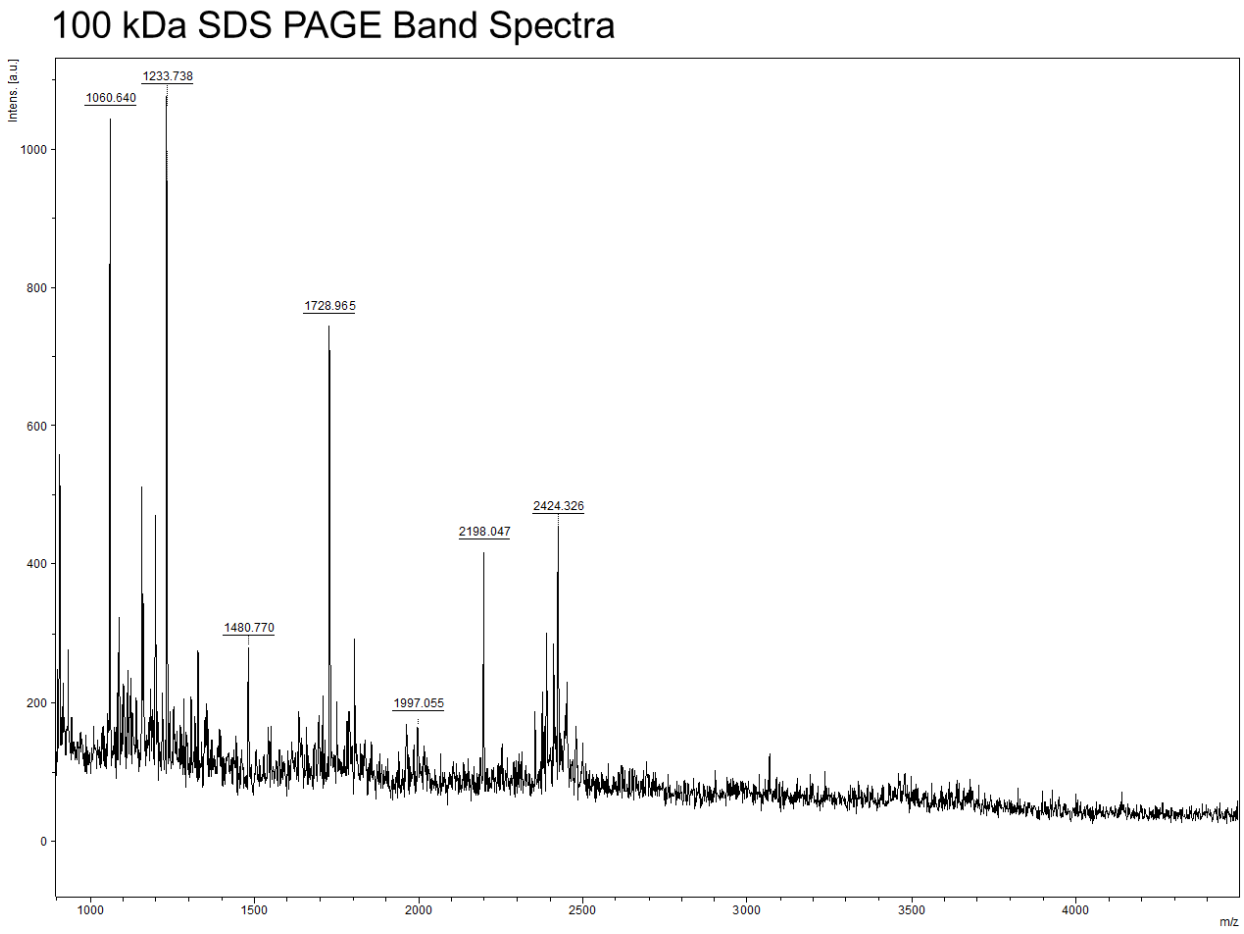


Figure A.1. 100 kDa SDS PAGE Band

Table A.1. 100 kDa SDS PAGE Band Identified Peptides

Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
1162.6810	1161.6737	1161.6295	38.1	1	-.RLSFYVGLAH.-
1328.8170	1327.8097	1327.8704	-45.7	1	R.IIFLVLRSLVR.I
1480.7700	1479.7627	1479.8231	-40.8	1	R.IPLAPMGPRSNSLK.I
1997.0550	1996.0477	1995.9890	29.4	1	K.MPWFFFRPENVSQRR.H
1750.9480	1749.9407	1749.9964	-31.8	0	R.QILLIHHLEPCPIPK.Q
1728.9650	1727.9577	1727.9830	-14.6	1	-.FAVGRLLLLPLMPASM.-
1198.7790	1197.7717	1197.6865	71.2	0	R.LLLLPLMPASM.-

52 kDa

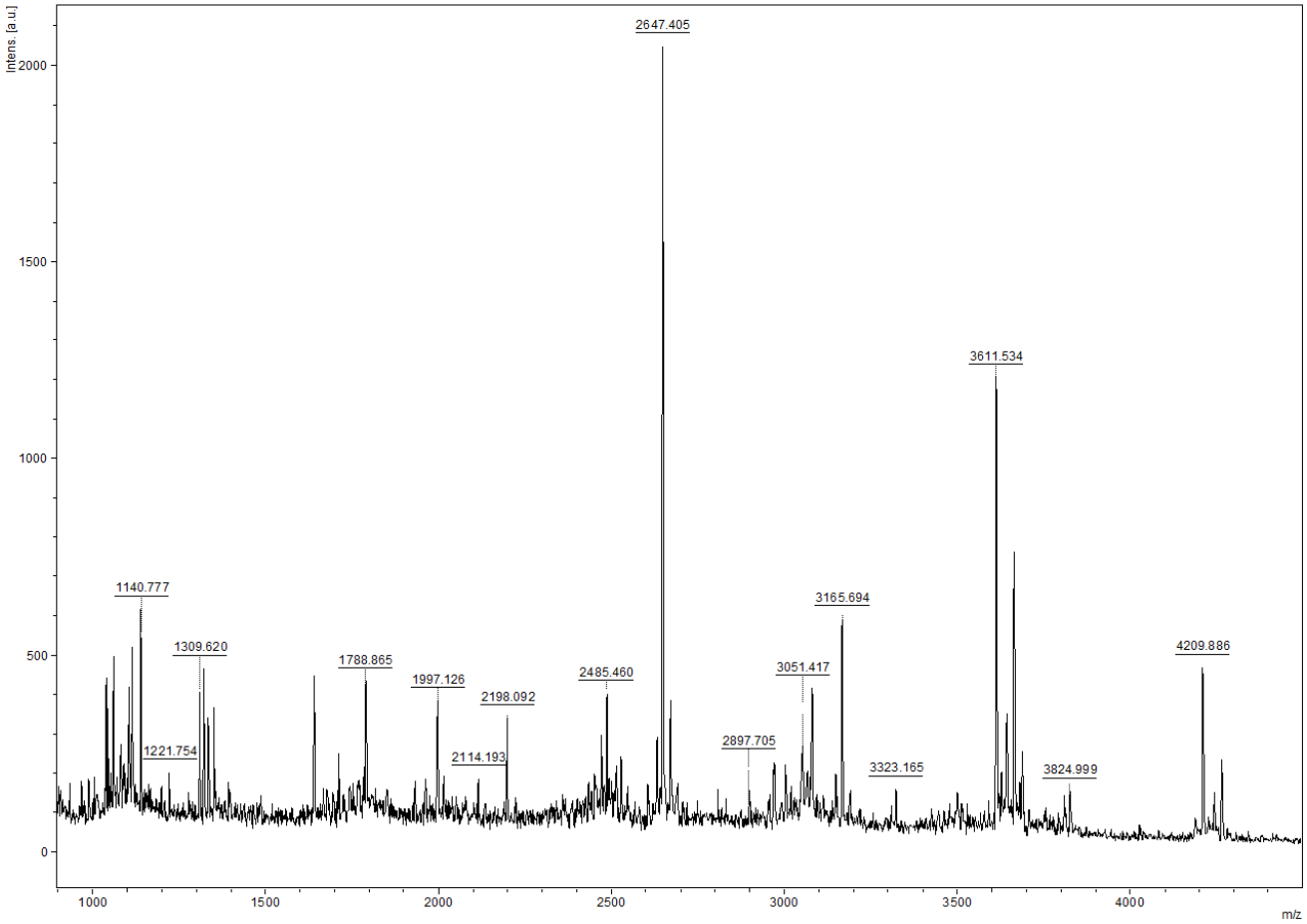


Figure A.2. 52 kDa SDS PAGE Band

Table A.2. 52 kDa SDS PAGE Band Identified Peptides

Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
1351.6680	1350.6607	1350.6667	-4.40	1	K.KDAEEYLGGEIK.R
1997.1260	1996.1187	1995.9836	67.7	1	R.AVITCPAYFNDAQRQATK.E
3079.4620	3078.4547	3078.6634	-67.8	1	R.KPIEQALSDAKLKPEDIDEIILVGGMTR.V
2969.4780	2968.4707	2968.6129	-47.9	1	K.LKPEDIDEIILVGGMTRVPMIQNFIK.E
3003.4550	3002.4477	3002.6818	-77.9	1	R.VFQGERPIAADNILLGSFRLVGIPPAPR.G
2897.7050	2896.6977	2896.4819	74.5	1	R.GVPQIEVTFDIDSDGIVHVSAKDLGTGK.E
1321.6550	1320.6477	1320.6197	21.2	1	K.EYGDKIPQDEK.Q
1309.6200	1308.6127	1308.6384	-19.6	1	K.MLFDELEREK.T
1114.5510	1113.5437	1113.6070	-56.8	1	K.TKIGEYIYK.Q

32-33 kDa

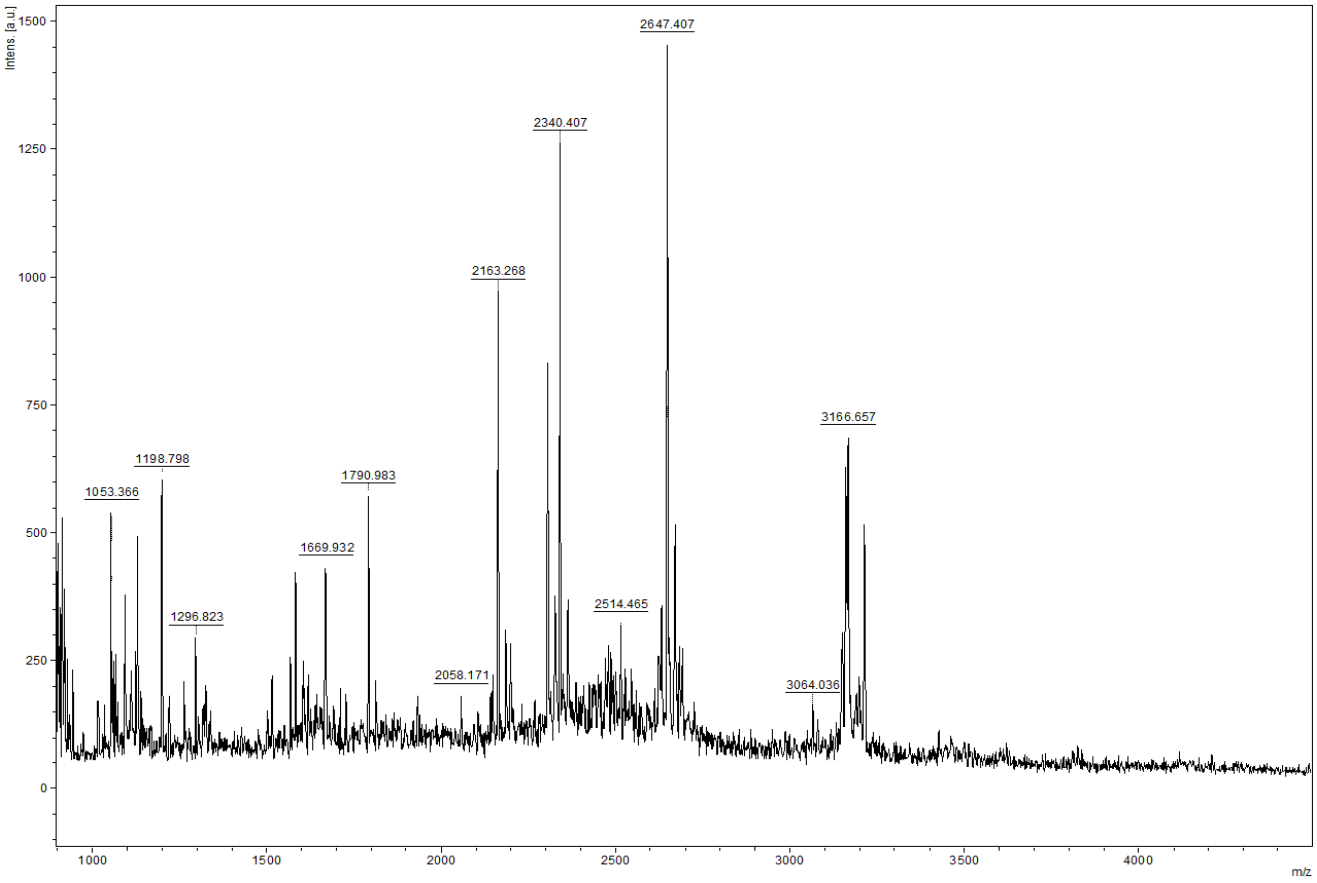


Figure A.3. 32-33 kDa SDS PAGE Band

Table A.3. 32-33 kDa SDS PAGE Band Identified Peptides

Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
2485.3490	2484.3417	2484.3370	1.92	1	R.QIAAGAIGITAAKISEAEVMASGGIR.D
902.3920	901.3847	901.4505	-73.0	0	R.EGAELEVR.L
1814.0700	1813.0627	1812.9581	57.7	1	R.EGAELEVRLELETGLR.R
2185.2470	2184.2397	2184.1361	47.4	1	K.LSGIFTYRGAMLGGASTLDVR.A
826.3410	825.3337	825.3729	-47.5	0	R.AAGHEEGR.L
1790.9830	1789.9757	1789.9250	28.3	0	K.TFATDIQPDNAPLFLK.G
2198.2260	2197.2187	2197.1718	21.4	0	R.IIPNHICSTVNLHSFVYIK.E

25 kDa

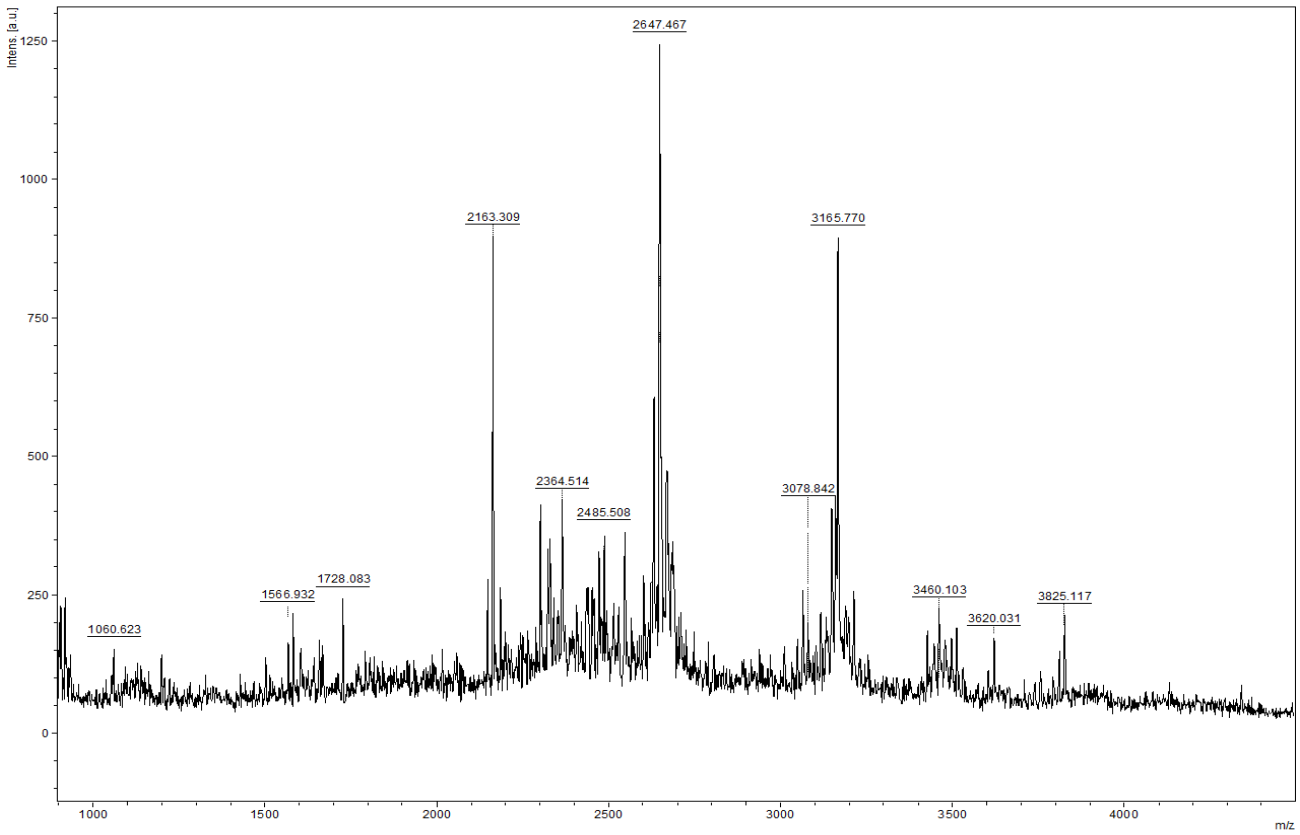


Figure A.4. 25 kDa SDS PAGE Band

Table A.4. 25 kDa SDS PAGE Band Identified Peptides

Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
804.3400	803.3327	803.3959	-78.7	0	-.MAEAVQR.C
1060.6230	1059.6157	1059.5495	62.5	1	M.AEAVQRCGVK.L
2485.5080	2484.5007	2484.3370	65.9	1	R.QIAAGAIGITAAKISEAEVMASGGIR.D
902.4230	901.4157	901.4505	-38.6	0	R.EGAELEVR.L
759.4330	758.4257	758.4286	-3.84	0	K.QISGLNK.I
2185.2320	2184.2247	2184.1361	40.6	1	K.LSGIFTYRGAMLGGASTLDVR.A
826.3420	825.3347	825.3729	-46.3	0	R.AAGHEEGR.L
1791.0100	1790.0027	1789.9250	43.4	0	K.TFATDIQPDNAPLFLK.G

15 kDa

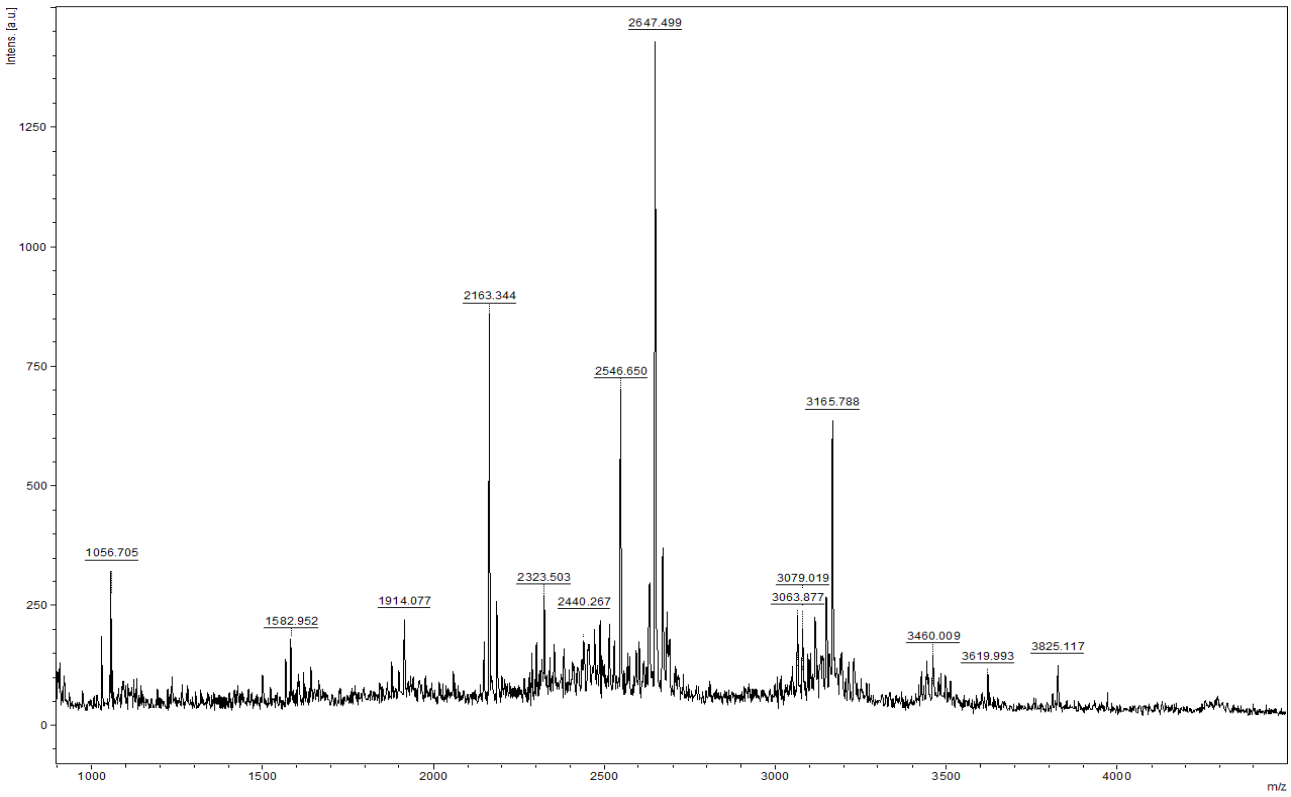


Figure A.5. 15 kDa SDS PAGE Band

Table A.5. 15 kDa SDS PAGE Band Identified Peptides

Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
2669.5660	2668.5587	2668.3895	63.4	1	R.LFDLDLLRAIVTVADCGSFTTAATR.L
2300.3690	2299.3617	2299.2457	50.5	0	R.LLALNDEMLEALSGATVALTVR.I
2382.3750	2381.3677	2381.2566	46.7	1	R.NPCIDLDPLPIVTFPPRGVYR.D
3825.1170	3824.1097	3824.0407	18.0	1	R.AVTADHQVLSRTTGLPAVDVFEVALLHRPAADPMVK.E
2647.4990	2646.4917	2646.4203	27.0	0	R.TTGLPAVDVFEVALLHRPAADPMVK.E
3116.8070	3115.7997	3115.6852	36.8	1	R.TTGLPAVDVFEVALLHRPAADPMVKELAR.V

13 kDa

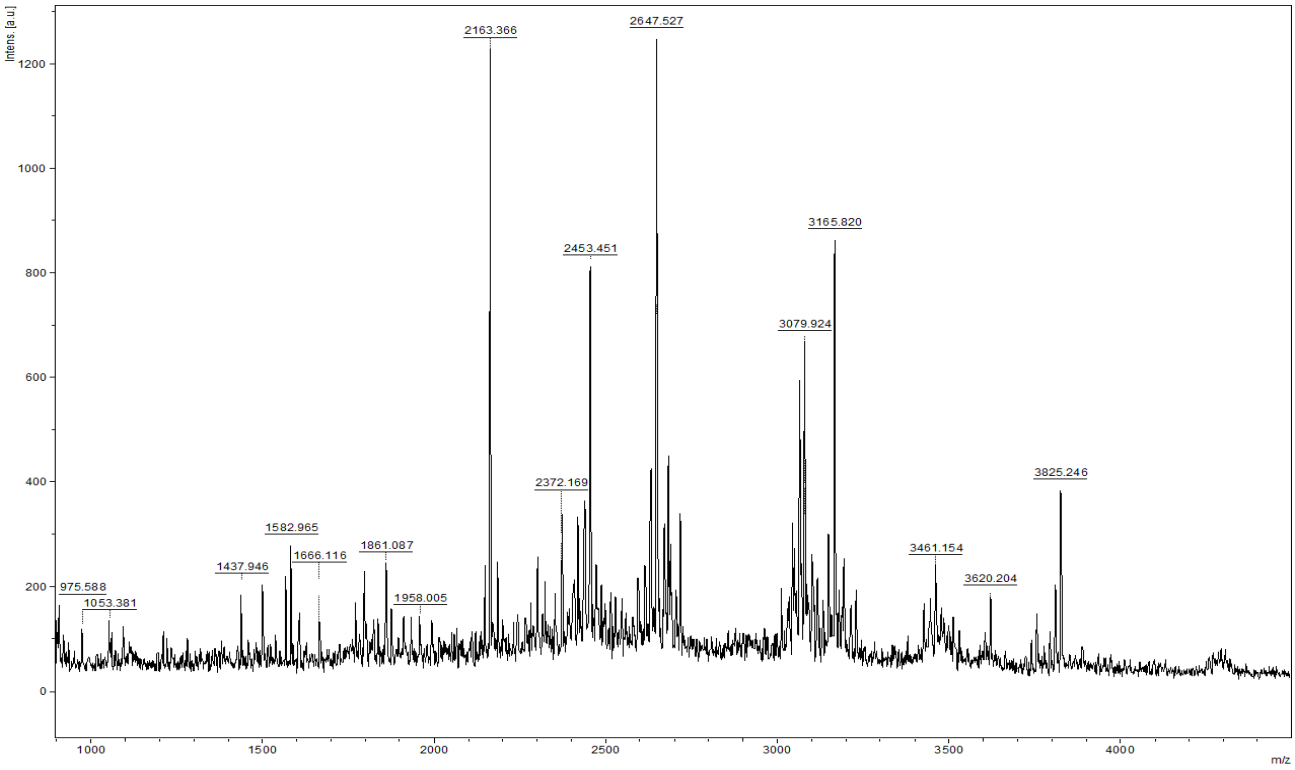


Figure A.6. 13 kDa SDS PAGE Band

Table A.6. 13 kDa SDS PAGE Band Identified Peptides

Observed	Mr(expt)	Mr(calc)	ppm	M	Peptide
3100.7720	3099.7647	3099.6083	50.5	1	K.DETEGATSMVVDMLSLLDAPTVVAAVPKIK.L
2185.3520	2184.3447	2184.2135	60.1	1	K.DFWFEILLNRLLFAIFK.R
1932.2190	1931.2117	1931.1105	52.4	1	-.GLARLVAFANLLNVSFAR.M
2647.5270	2646.5197	2646.5036	6.09	0	K.APLILEPDPQLLLNNLPFLEFLK.F
3079.9240	3078.9167	3078.7157	65.3	1	K.APLILEPDPQLLLNNLPFLEFLKFER.H
848.3590	847.3517	847.4044	-62.2	0	-.SICCLGPR.L

A.2. MASCOT Peptides Identified

Amino Acid Analysis MASCOT Protein Prediction 12/6/17

100 kDa

XP_010169331.1 Mass: 17237 Score: 99 Expect: 0.016 Matches: 11

PREDICTED: centriole, cilia and spindle-associated protein, partial [Antrostomus carolinensis]

XP_010308853.1 Mass: 17267 Score: 99 Expect: 0.016 Matches: 11

PREDICTED: centriole, cilia and spindle-associated protein, partial [Balearica regulorum gibbericeps

52 kDa

WP_015161582.1 Mass: 16647 Score: 76 Expect: 3 Matches: 7

hypothetical protein [Chamaesiphon minutus]

32 kDa

WP_035329257.1 Mass: 106667 Score: 76 Expect: 3.2 Matches: 14

excinuclease ABC subunit UvrA [Bacillus firmus]

25 kDa

ESQ13940.1 Mass: 21517 Score: 79 Expect: 1.8 Matches: 8 hypothetical protein N838_13390 [uncultured Thiohalocapsa sp. PB-PSB1]

15 kDa

WP_058791284.1 Mass: 78317 Score: 84 Expect: 0.57 Matches: 13

mechanosensitive ion channel family protein [Pseudomonas psychrotolerans]

13 kDa

AFC45875.1 Mass: 49273 Score: 67 Expect: 26 Matches: 10

DoxD family protein/pyridine nucleotide-disulfide oxidoreductase [Mycobacterium intracellulare ATCC 13950]

AGP66229.1 Mass: 49273 Score: 67 Expect: 26 Matches: 10

DoxD family protein/pyridine nucleotide-disulfide oxidoreductase [Mycobacterium yongonense 05-1390]

EUA28879.1 Mass: 49286 Score: 67 Expect: 26 Matches: 10

pyridine nucleotide-disulfide oxidoreductase family protein [Mycobacterium avium subsp. avium 2285 (S)]

WP_009952488.1 Mass: 49908 Score: 67 Expect: 28 Matches: 10

MULTISPECIES: NAD(P)/FAD-dependent oxidoreductase [Mycobacterium]

WP_008261470.1 Mass: 49952 Score: 67 Expect: 28 Matches: 10

NAD(P)/FAD-dependent oxidoreductase [Mycobacterium sp. H4Y]

WP_038537842.1 Mass: 49921 Score: 67 Expect: 28 Matches: 10

Table A.7. MALDI TOF-MS peptides identified in SDS-PAGE 13 kDa band

13 kDa band	DoxD family protein/pyridine nucleotide-disulfide oxidoreductase	pyridine nucleotide-disulfide oxidoreductase family protein	MULTISPECIES: NAD(P)/FAD-dependent oxidoreductase	
	67%	28%	5%	100%

Table A.8. MALDI TOF-MS peptides identified in SDS-PAGE 15 kDa band

15 kDa band	LysR transcriptional regulator	Mechanosensitive ion channel family protein	LysR substrate binding domain protein
	89%	10%	1%

Table A.9. MALDI TOF-MS peptides identified in SDS-PAGE 15 kDa band

25 kDa ba	Uncharacterized protein	ATP-dependent Clp protease ATP-binding subunit ClpX	Glutamine synthetase	Methyl-accepting chemotaxis sensory transducer	Acetyltransferase	UvrABC system protein C (Protein UvrC) (Excinuclease ABC subunit C)	2-dehydro-3-deoxyglu conokinase	Amino acid aldolase	GTPase Era	Oxidoreductase	RNA-binding transcriptional accessory protein	Tex-like protein N-terminal domain protein	Type I-C CRISPR-associated protein Cas8c/Csd1	Alpha/beta hydrolase	Cell division protein	Histidine kinase	N-ethylammeline chlorohydrolase
	29%	28%	6%	5%	5%	5%	3%	3%	3%	3%	2%	2%	2%	1%	1%	1%	1%

Table A.8. MALDI TOF-MS peptides identified in SDS-PAGE 32 kDa band

32 kDa ba	ATP-dependent Clp protease ATP-binding subunit ClpX	Amino acid aldolase	Uncharacterized protein	Tryptophan synthase alpha chain (EC 4.2.1.20)	His Kinase A (Phospho-acceptor) domain-containing protein	EC 3.6.1.1 Membrane-bound sodium-translocating pyrophosphatase	D-serine deaminase-like pyridoxal phosphate-dependent protein	2Fe-2S ferredoxin	Low-specificity D-threonine aldolase	Catalytic	Ferredoxin-NADP reductase	Acetate--CoA ligase	Ferredoxin 1
	62%	9%	7%	2%	2%	2%	2%	2%	2%	2%	6%	1%	1%

Table A.10. MALDI TOF-MS peptides identified in SDS-PAGE 52 kDa band

52 kDa band	Chaperone protein DnaK (HSP70) (Heat shock 70 kDa protein)	DnaK (Fragment)	Heat shock protein 70 mitochondrial putative	Uncharacterized protein
	80%	12%	7%	1%

Table A.11. MALDI TOF-MS peptides identified in SDS-PAGE 100 kDa band

100 kDa band		
	centriole, cilia and spindle-associated protein, partial	hypothetical protein H355_011766
	99%	1%

Vita

Chelsea Tyus is an only child from St. Louis, MO. She graduated high school from Lutheran High School North in St. Louis, MO in 2005. She received a Bachelor of Science from Alabama A&M University in Food Science and Technology in 2009. In 2014, Chelsea received a Master of Science from University of Missouri in Food Science with emphasis in Food Chemistry. Chelsea worked as a pharmacy technician, executive assistant, quality assurance technician, regulatory affairs specialist, teaching/research assistant while in school. In 2019, Chelsea graduated from Louisiana State University with a Doctorate in Food Science. She specializes in macronutrient extraction, macronutrient characterization and chromatography. She has researched grape pomace, sweet potato, microalgae, rice flour, and sorghum flour. Chelsea has presented poster's at IFT National Conference (2018, 2019), ABO Summit (2018, 2019), and AOCS National Conference (2019). Chelsea is working on several publications for the Journal of Algae Research and the Journal of Food Science. Chelsea's personal goal is to help the public understand and, as a result, consume healthy and safe food. Chelsea is endeavoring to obtain an instructor position at a university or scientist position in a food/chemical company. Chelsea enjoys traveling, reading yoga in her spare time.